Studies on the molecular interactions of collagen type XVI

Part I:

The role of collagen XVI in pathological disorders

Part II:

Establishment of a retroviral mediated gene silencing model

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By

Sabine Ratzinger

from Unterötzdorf

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Under the supervision of   Prof. Dr. Roland Seifert

                      and

             PD Dr. Susanne Grässel

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                             1. reviewer: Prof. Dr. Roland Seifert

                             2. reviewer: PD Dr. Susanne Grässel

                             3. examiner: Prof. Dr. Achim Göpferich

"You have to love what you do, do what you have to do, you gotta work hard, as hard as you can, to move the stones out of your way."

Lyrics from "breathe" by Mohear

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Acknowledgements

Abbreviations

APS	Ammoniumperoxodisulfate solution
Amp	ampicillin
BCA	bichinonic acid
bp	base pair
BSA	bovine serum albumin
Calcein AM	calcein acetoxymethylester
cDNA	complementary DNA
CD	crohn's disease
Cfu	colony forming units
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid
CMV	cytomegalovirus
COL	collagenous domain
COMP	cartilage oligomeric protein
CPD	critical point drying
Ct	threshold cycle
DAPI	4', 6'-diamidino-2-phenylindole, dihydrochloride
DEJ	dermo-epidermal junction
DMEM	dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	mixture of desoxyribonukleotides dATP, dCTP, dGTP, dTTP
dsRNA	double stranded RNA
DTT	dithiothreitol
E. coli	Escherichia coli
ECCO	European crohn's and colitis organization
ECM	extracellular matrix
EDTA	ethylendiamintetraacetate
EMEM	eagle's modified essential medium
ELISA	enzyme-linked immunosorbant assay
et al.	et alii (lat.: and others)
EtD1	ethidium homodimer
ex/em	extinction/emission
FA	focal adhesion

FACIT	fibril associated collagen with interrupted triple helices
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FC	focal contact
FCS	fetal calf serum
FN	fibronectin
FN ED A	fibronectin extra domain A
FN ED B	fibronectin extra domain B
g	gravitation
G418	geniticin
HEPES	N-2-Hydroxyethylenpiperazin-N'-2-ethansulfon acid
IBD	inflammatory bowel diseases
IEF	isoelectric focussing
IEP	isoelectric point
IgG	gamma immunoglobulin
IPG	immobilized polyacrylamid gel
ISEMF	intestinal subepithelial myofibroblasts
kb	kilo bases
kDa	kilo Dalton
LB	Luria-Bertani
mAb	monoclonal antibody
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time Of Flight
max.	maximum
MCS	multiple cloning site
MIF	macrophage migration inhibitory factor
MIDAS	metal ion dependent adhesion site
MMP	matrix metalloproteinase
miRNA	microRNA
mRNA	messenger ribonucleic acid
MW	molecular weight
NC	non-collagenous domain
nt	nucleotide
OD	optical density
PA	polyacrylamide

PAGE	polyacrylamide gel electrophoresis
PARP	prolin-arginin-rich protein sequence
PBS	phosphate buffered saline
PBST	0.1 % Tween 20 in PBS
PCR	polymerase chain reaction
PFA	para-formaldehyde
PPIA	prolylpeptidyl-cis-trans-isomerase A
qPCR	quantitative polymerase chain reaction
rct	reaction
RISC	RNA-induced silencing complex
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
rpm	rotations per minute
RQ	relative quantitation value
rt	room temperature
hr	hour
hrs	hours
sec	second
SDS	sodium dodecyl sulphate
SEMF	subepithelial myofibroblasts
SFM	serum-free medium
SEM	scanning electron microscopy
shRNA	short hairpin loop containing RNA
SOC	super optimal broth with catabolite repression
tab.	Table
TAE	tris-acetate EDTA
taq	Therophilus aquaticus
TCA	trichloric acid
TEM	transmission electron microscopy
TEMED	triethylmethylethylendiamin
TGF-ß	Transforming growth factor beta
TNF-α	Tumor-necrose-factor alpha
Tris	Tris(hydroxymethyl)-aminomethan

TSPN	thrombospondin
UC	ulcerative colitis
w/o	with or without
v/v	ratio of volumes
w/v	ratio of weight to volume

1. Abstract

Molecular interactions of collagen XVI in Crohn's disease

In Crohn's disease (CD) the stress-shield of intestinal subepithelial myofibroblasts (ISEMF), provided by intact tissue is disturbed due to inflammation and cells start with remodelling activities. This is characterized by increased numbers of collagen-producing ISEMF causing an uncontrolled, irreversible wound-healing response to the chronic inflammation of the gastrointestinal tract. Reconstitution of the original extracellular matrix (ECM) leads ISEMF to exit this cycle, however, in fibrosis ISEMF remain. It is known that ISEMF produce and deposit collagen types I, III, IV and V; but synthesis and the role of fibrillar peripheral molecules like collagen type XVI have not been addressed yet. Here, we have analyzed distribution of collagen XVI in the normal and inflamed bowel wall, its gene and protein expression by ISEMF of different inflammation stages, the cell-matrix interactions in different phases of the inflammatory process and the effect of collagen XVI on cell proliferation and migration. Collagen XVI is deposited in the submucosa of the intestinal wall and ISEMF reveal increasing gene and protein expression of collagen XVI with concurrent increasing inflammation. ISEMF display more mature focal adhesion contacts when seeded on collagen XVI resulting in an extensive cell spreading. This might involve recruitment of α1 integrin, since its cell surface expression on ISEMF is increased in late stages of inflammation. We assume that collagen XVI promotes persistence of ISEMF in the normal and even stronger in the inflamed bowel wall by stabilizing focal adhesion contacts via cell-matrix interaction preferentially through recruitment of α1ß1 integrin into the focal contacts.

Mechanistical studies of collagen XVI by a retroviral mediated knockdown model in murine fibroblasts

Collagen XVI, a member of FACIT collagens (fibril associated collagens with interrupted triple helices) is described as macro-molecule of the ECM. Very little is known about its role in cell-matrix-interactions and in cell signalling. However it has been demonstrated that cells interact with collagen XVI via integrin α1β1 and α2β1. These interactions presumably determine organization of extracellular components and their communication with cells. We have established a retroviral mediated collagen XVI knockdown in NIH3T3 fibroblasts to investigate the role of collagen XVI in cell-matrix interactions. Specific shRNAs against collagen XVI were utilized in parallel to a viral

luciferase vector construct as control. After successful transduction, positive cell clones were further selected by antibiotic resistance. Knockdown efficiency was determined on mRNA and protein level and further downstream experiments were performed with respect to adhesion and proliferation. Differential protein expression in knockdown cells was compared to the control by 2D-gelelectrophoresis.

The knockdown resulted in 80-90 % inhibition of collagen XVI gene and protein expression and mass spectrometry revealed several differentially expressed proteins. Collagen XVI inhibited cell lysates showed a lack of macrophage migration inhibitory factor (MIF) and prolylpeptidyl-cis-trans-isomerase A (PPIA). The gene expression of these proteins was slightly up-regulated, however, western blot analysis confirmed 2D-gelelectrophoresis results. Proliferation of knockdown cells was generally reduced and was not influenced by the presence of collagen XVI as cell culture substrate. The cytokine MIF influences migration and proliferation of fibroblasts during wound healing. MIF induces synthesis of collagens in fibroblasts (collagen I, III, IV, V and VI) whereas PPIA contributes to correct protein folding in the cytoplasm of cells. Collagen XVI acts as adapter molecule in organizing suprastructures. Therefore, we assume that the lack of collagen XVI detains fibroblasts in arranging fibrillar stuctures which results in disturbed initial adhesion. The amelioration of cellular adhesion to the available matrix indicates a compensation in integrin expression pattern. Reduced proliferation together with decreased MIF expression hints at changes in the differentiation stage and has to be further elucidated.

2. Preface

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka' (I found it!) but 'That's funny…' Isaac Asimov

2.1. Molecular constituents of the ECM

Multicellular organisms comprise of ingenious arrangements of cells and surrounding molecules. Structural integrity and functionality in tissues are determined by a highly organized architecture of extracellular matrix generated by residing cells and culminates in organ development. The extracellular matrix is not only giving shape and mechanical resilience in a tissue, it is also directly interacting with cells and this form of communication is influencing cell differentiation, proliferation, adhesion and migration. The function of extracellular matrix exceeds simple mechanical tasks and is involved in complex cell-matrix interactions.

The extracellular matrix comprises of collagens, proteoglycans and further macromolecular components. These constituents and their composition vary according to tissue-specific or evolutionary requirements.

2.1.1. Collagens – a family portrait

Around 30 % of protein in all tissues are collagens, the most abundant proteins in mammals. Collagens are glycoproteins that are characterized by a triplehelical structure enabled by a specific repetitive amino acid sequence: $(Gly-Xaa-Ybb)_n$. Glycine is located in the inner part of the clockwise turning helix and Xaa and Ybb are often allocated by proline that stabilizes the triple-helical structure if hydroxylized. There is a huge complexity and diversity of the 29 identified collagens that form various supramolecular structures.

The biosynthesis of collagens is basically characterized for fibrillar collagens that underlie co- and posttranslational modifications. The intracellular modifications require at least 9 hydroxylases and glycosyltransferases (Lamande and Bateman, 1999) and comprise hydroxylation of some proline and lysine residues and the glycosylation of several hydroxylysine and asparagine residues. This is occurring sequentially in the rough endoplasmic reticulum whereas the Golgi apparatus is mediating the association of a stable triple helix from the C-terminus towards the N-terminus. The resulting soluble pro-collagen is secreted into the extracellular space and proteolytically cleaved.

The separation of N- and C-propeptides is leading to an insoluble collagen molecule that can spontaneously assemble to collagen fibrils (Birk and Silver, 1984; Jones et al. 1998; Richard-Blum and Ruggiero, 2005).

Collagens differ extremely in their complex structural and functional characteristics. Therefore, the term collagen underlies some simplifying criteria: The protein has to be found in the extracellular space and has to contribute to structural integrity of the ECM. It must contain at least one triple-helical domain and assemble in supramolecular structures, either alone or together with other matrix components (Jacenko et al. 1991). According to their amino acid sequence, their molecular composition, and their function, collagens are divided into several subfamilies (Table 2-1). Basically there are two major groups: fibrillar and non-fibrillar collagens. Fibrillar collagens comprise collagen type I, II, III, V, XI, XXIV, XXVII and are able to form stable fibrils. They represent around 90 % of all collagens found in mammals and share homologies in their molecular structure. A collagenous domain of around 1000 amino acids represents the middle part of an alpha chain, framed by a highly conserved non-collagenous C-terminal and a variable N-terminal domain. Procollagens assemble as homo- or heterotrimers and after cleavage of N- and C-terminal propeptides the processed collagen-trimers form around 300 nm long triple helices: the collagen fibrils (Bornstein, 1974; Prockop and Kivirikko, 1995). The aggregation is laterally staggered, therefore, in transmission electron microscopy with heavy metal contrast 63-67 nm periodically D-banded fibrils occur. Dependent on the tissue and the localization in the tissue the fibrils vary in their size and their heterogenous composition.

Non-fibrillar collagens are divided into several subgroups. Collagen IV and VII are basement membrane collagens that are grouped by their molecular structure but form different aggregates. Collagen VII builds anchoring fibrils, whereas collagen IV assembles in C-terminal aggregated net-like tetramers. Together with these collagens also the multiplexin-collagens type XV and XVIII occur in the basement membrane. Collagen VI, VIII and X are grouped in the family of short collagens that share the feature of just one collagenous domain framed by huge non-collagenous domains. The short collagens assemble in different tissues as hexagonal networks. Transmembrane collagens are represented by collagen type XIII, XVII, XXIII and XXV. They form homotrimers with an N-terminal intracellular domain, a hydrophobic transmembrane part and an extracellular C-terminus that varies in a different number of triple helical domains (Franzke et al., 2003).

Collagen family	Family member	Integrin binding sites identified for:
Fibril-forming collagens	Collagen I, II, III, V, XI, XXIV, XXVII	**Collagen I,II,III, V monomers** α2β1, α1β1, α10β1, α11β1 **Collagen XI monomers** α2β1 **Collagen I fibrils** α2β1, α11β1 **Denatured collagen I** α5β1, αVβ1 **C-propeptide in collagen I and II** α2β1
FACITs (fibril associated collagens with interrupted triple helices)	Collagen IX, XII, XIV, XVI, XIX, XX, XXI, XXII	**Collagen IX** α2β1, α1β1, α10β1, α11β1 **Collagen XVI** α2β1, α1β1
Hexagonal networks forming collagens	Collagen VIII, X	**Collagen X** α2β1 **Collagen VIII** α2β1, α1β1
Basement membrane collagen	Collagen IV, VII	**Collagen IV** α1β1, αVβ3, αVβ5, α3β1
Beaded-filaments forming collagen	Collagen IV	**Collagen IV** α1β1, α10β1, α2β1, α11β1
Anchoring fibrils forming collagen	Collagen VII	**Collagen VII** α2β1
Transmembrane collagen	Collagen XIII, XVII, XXIII, XXV	**Collagen XIII** α1β1 **Collagen XVII** α5β1, αVβ
Multiplexins	Collagen XV, XVIII	**Collagen XVIII** α5β1, αVβ3, αVβ5

Table 2-1: Collagen types and interacting integrins (Heino, 2007).

2.1.2. The FACIT- collagen type XVI

The largest group of collagens is the family of FACIT-collagens. The first discovered members collagen IX, XII, XIV were associated to fibrillar collagens, therefore, they were termed **f**ibril **a**ssociated **c**ollagens with **i**nterrupted **t**riple helices (FACIT) that do not form fibrils on their own (Gordon et al., 1989; Richard-Blum et al., 2000). Collagen IX, for example, is covalently bound to the fibril surface and was proven to act as a linker protein between cartilage fibrils and other matrix components (Budde et al.,

2005). The function of most FACITs could not be elucidated completely, however, it has been proposed that these collagens play a role in stablizing and/or in organizing fibrillar networks in the extracellular matrix. Although FACITs vary in their size and exhibit a huge structural diversity they all share common structural features. Two highly conserved cysteine residues are separated by four amino acids in the junction between the first collagenous (COL) and the first non-collagenous (NC) domain and two G-X-Y triplet imperfections in the first collagenous domain. The collagens IX, XII, XIV, XVI, XIX, XX, XXI and XXII were grouped according to these conserved features. FACIT-collagens are further characterized by collagenous amino acid sequences that are interrupted and flanked by non-collagenous domains. The domains are sequentially numbered from the C-terminal part. Some non-collagenous domains display homologies to functional parts of other matrix proteins. All FACIT-collagens harbour a proline-arginin-rich protein sequence (PARP) that shows similarities to thrombospondin (TSPN). Collagen type XII, XIV and XX also display in their N-terminus several von-Willebrandt-Factor-A-domains (VA-domain) and some fibronectin-III-domains (F3) (Richard-Blum et al., 2000).

In 1992 the cDNA sequence of collagen XVI has been discovered in a screening of cDNA banks for collagen-like sequences (Pan et al., 1992; Yamaguchi et al., 1992). The cDNA of 5.4 kb comprises a 4809 bp coding sequence, framed by non-translated parts, including a 425 bp 3'-non-coding sequence. Collagen XVI has been localized on chromosome 1, 1p34-35 (Pan et al., 1992). The coding sequence of collagen XVI comprises of 1604 amino acids including a 21 amino acid signal peptide, whereas the recombinant version of the protein has 1597 amino acids (Kassner et al., 2004). Collagen XVI appears as homotrimer of three identical alpha1-chains each consisting of 10 collagenous and 11 non-collagenous domains. The 312 amino acid N-terminus harbours the PARP-motif, the TSPN-domain. The typical conserved imperfections in the intersection of COL1 and NC1, the Cys-Xaa-Ybb-Cys-motif is also found in collagen XVI and assigns this protein to the FACIT-family of collagens. Collagen XVI contains seven of these imperfections.
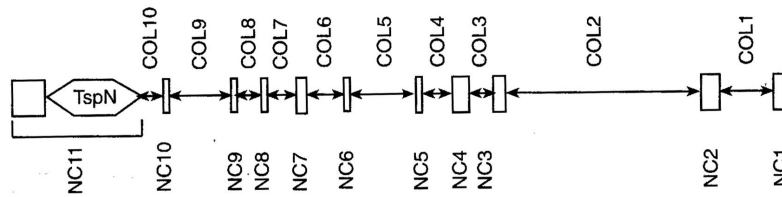
Figure 2-1: The domain structure of collagen XVI (Pan et al., 1992; Richard-Blum et al., 2000).

COL: collagenous domains, NC: non-collagenous domains, TspN: Thrombospondin-like domain. The N-terminus harbours the TSPN-domain. Each of the three identical alpha1-chains forming a homotrimer consists of 10 collagenous and 11 non-collagenous domains.

Collagen XVI comprises three identical alpha1-chains each of around 200-220 kDa according to globular protein standards. The full length protein is proteolytically cleaved with respect to the expressing cell type, without any hint of splice variants. Polypeptide antibodies targeting the NC11-domain detect cleavage products of 40 kDa, 65 kDa and 70-90 kDa in the cell culture supernatant of fibroblasts (Akagi et al., 1999; Tajima et al., 2000). Quantified with the same antibody, medium of fibroblasts and medium and cell lysate of dendrocytes showed peptides of 220 kDa, 180 kDa, 130 kDa and 62 kDa (Akagi et al., 2002). After immuno-precipitation cell lysates and cell culture supernatants of smooth muscle cells and dermal fibroblasts displayed protein fragments of 220 kDa, 180 kDa, 150 kDa, 130 kDa and 75 kDa (Grassel et al., 1996; Grassel et al 1998).

Recombinant collagen XVI was over-expressed in an episomal system in HEK 293 EBNA cells and secreted into the cell culture medium. A C-terminal Strep-Tag allowed the purification of collagen XVI by affinity chromatography. The protein was characterized by N-glycosylated alpha1 chains assembled in trimers that were attached by disulfide bonds.

The N-terminal sequencing of recombinant collagen XVI could identify several cleavage products (213 kDa, 182 kDa, 133 kDa, 78 kDa) with their specific cleavage sites (Kassner et al., 2004). The sequence of the full length protein with 213 kDa comprises the BM40 signal peptide followed by the collagen XVI sequence. The 182 kDa fragment starts at amino acid 257 and lacks the NC11 domain cleaved within the

sequence KARRD between R(256) and D(257). This sequence contains a furin cleavage recognition site (Tillet et al., 1995). The 78 kDa fragment comprises COL1 and COL2 with a cleavage site in the NC3 domain within the sequence AELG between E(941) and L(942). This amino acid sequence could be detected by aggrecanase-1 and -2, namely ADAM TS4 and ADAM TS5 (Tortorella et al., 2001). Collagen XVI appears in electron microscopy as flexible filamentous molecule with a clover-leaf shaped NC11 domain. The full length protein is of an average size of 240 nm and is posttranslational hydroxylized. It shows a decreased thermo-stability compared to authentic collagen XVI. Binding studies displayed a high affinity to fibronectin, whereas fibrillin-1 / -2 bind with a lower affinity to collagen XVI. None of these molecules shows any affinity to the recombinant NC11 domain (Kassner et al., 2004).

2.2. Molecular assemblies involving collagen XVI

Collagen XVI is expressed by keratinocytes (Grassel et al., 1999), fibroblasts and smooth muscle cells (Grassel et al., 1996), neuronal cells of the dorsal root ganglion (Hubert et al., 2007), chondrocytes (Kassner et al., 2003), and dendrodytes, a subgroup of dermal dendritic macrophages able to differentiate from blood monocytes after interleukin-4 stimulation (Akagi et al., 2002).

In the embryonic development of the mouse collagen XVI showed a tissue distribution comparable to fibrillar collagens, however, temporarily delayed. Collagen XVI was widely expressed except for skeletal muscles, lung, and brain. m-RNA expression started at day 11 in the heart followed by spinal nerve fibres, fibrous connective tissue of the liver, intestinal tissue and differentiated chondrocytes. It was also expressed in ovary, testis, eye, skin and smooth muscle cells which altogether indicates a mesenchymal origin and displays collagen XVI mainly in the neighbourhood of basement membranes (Lai and Chu, 1996).

In the human skin collagen XVI is expressed with respect to the cellular origin within the several layers of the skin. Collagen XVI is located close to basement membranes within the dermo-epidermal junction (DEJ) of the upper papillary dermis and around blood vessels. In the DEJ collagen XVI is co-localized with fibrillin-1 containing microfibrils however not in deeper dermis layers (Grassel et al., 1999). Fibroblasts that are non-adherent or confluent express less collagen XVI than cells of another phase of the cell cycle which is in contrast to the expression of collagen I sharing the same conditions (Tajima et al., 2000).

Skin fibroblasts and smooth muscle cells reacted to TGF-ß1 stimulation with an increase of collagen XVI expression. The stimulation also resulted in an augmented deposition of collagen XVI in the ECM, whereas FGF-2 repressed collagen XVI gene expression and biosynthesis (Grassel et al., 1998).

In human adult costal cartilage collagen XVI was identified as part of the ECM. Collagen XVI is localized in the peri-cellular area within the territorial matrix of chondrocytes, however, it is not co-localized with fibrillin-1 in cartilage tissue. Moreover, collagen XVI is associated with thin D-banded, collagen-containing cartilage fibrils (Kassner et al., 2003).

2.3. Cell-matrix adhesion via integrins

Integrins are structurally and functionally related heterodimeric cell surface receptors which mediate the adhesion of cells to the ECM. They are involved in regulatory processes, the embryonic development, angiogenesis and tumorigenesis. So far, 18 α-subunits and 8 β-subunits are known that form 25 integrin heterodimers (Fig. 2-2). Integrins link the actin cytoskeleton to the ECM and therefore convey information about localization and surrounding to the cell (Gullberg and Lundgren-Akerlund, 2002; Pfarrer et al., 2003). Integrins are grouped in various systems, according to a conserved structure, composition of their subunits or their ligand specifity. Via integrins the components of the ECM not only induce cellular reactions like cell adhesion, spreading, migration, and gene activation in an outside-in manner, moreover, ECM can also be remodelled by cells in an inside-out fashion (Akiyama et al., 1989; Aplin et al., 1998). Weak binding conformation of integrins is altered by internal signals into an active adhesive conformation and results in an interaction with the extracellular matrix. A loss of adhesion activates several caspases which in turn induce anoikis, a form of programmed cell death (Pankov et al., 2003; Stupack et al., 2001).
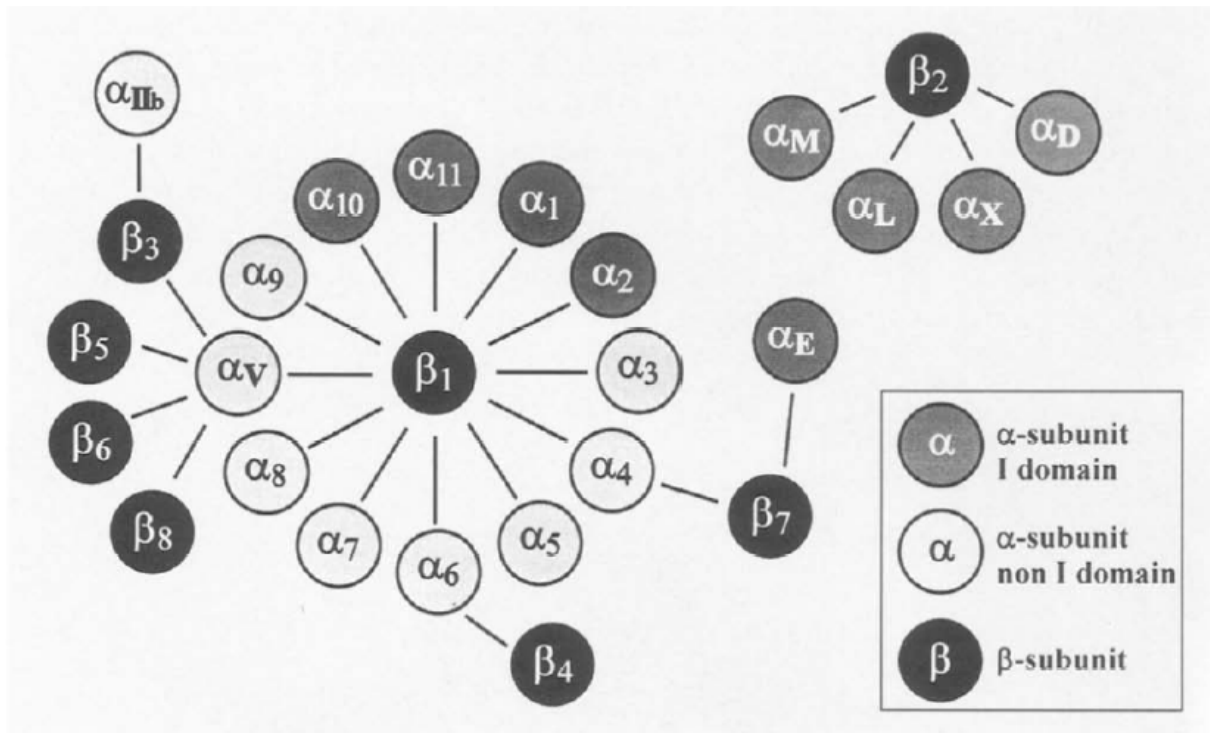
Figure 2-2: The integrin family.

18 α-subunits are able to interact with 8 β-subunits to form an active integrin complex. The α-subunits containing an I domain able to bind collagens are marked in dark grey (Gullberg and Lundgren-Akerlund, 2002).

2.3.1. Structure of integrins

Both subunits comprise a huge extracellular, a short transmembrane domain and a cytoplasmic part. α- and β-subunits are non-covalently bound and display a low ligand affinity. Therefore, cells quickly adhere via integrins however they can also easily loose this connection serving as basis for cell motility and invasion (Brakebusch and Fassler, 2003).

The α-subunit comprises a large extracellular domain composed of a globular head on a stalk bearing a sevenfold β-propeller (Fig. 2-3). The I domain is the obligate ligand binding site. However, not all α-chains contain interaction sites (Hynes, 2002; Springer, 1997). α-subunits without the I domain obtain their ligand specifity by a β-subunit bearing a highly conserved I domain, that is able to bind cations like Mn^{2+} or Mg^{2+} via a MIDAS (metal ion dependent adhesion site) motif. The binding of metal ions leads to a conformational change in the I domain that enables ligand binding (Humphries et al., 2004).

The cytoplasmic domain of both chains connects the extracellular matrix with the actin cytoskeleton and triggers intracellular downstream processes by recruiting structural and interconnecting proteins.
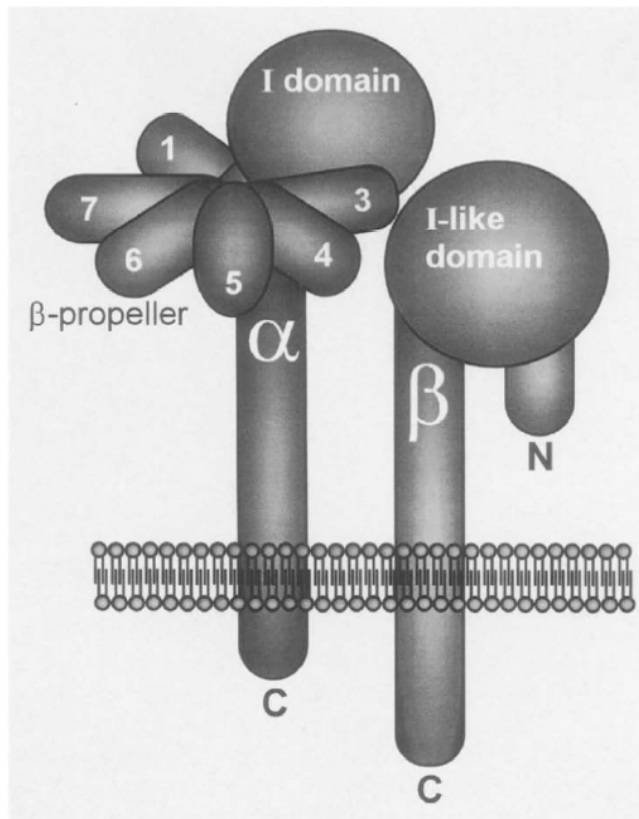
Figure 2-3: α- and β-subunits of integrins.

The α- and β-subunits comprise a large extracellular domain, a transmembrane domain and short cytoplasmic tail. The β-propeller, which is able to bind bivalent cations, is located at the N-terminus of the α-chain. The β-subunit contains a N-terminal located I domain, that binds metal ions (Gullberg and Lundgren-Akerlund, 2002).

2.3.2. Collagen-binding integrins

So far four collagen binding integrins are known, namely α1β1, α2β1, α10β1, α11β1 (Camper et al., 1998; Gullberg and Lundgren-Akerlund, 2002; Velling et al., 1999). Studies on collagen-binding integrins *in vitro* show their activity and influence in cell adhesion, cell migration, control of collagen synthesis, matrix metalloproteinase (MMP) synthesis, remodelling of collagen matrices, cell proliferation, cell differentiation and angiogenesis (Bouvard et al., 2001). Important functions could be indentified *in vivo*: reorganization of collagen matrices within developing tendons, ligaments, periosteum, capsules of organs and in healing wounds (Stopak and Harris, 1982).

2.3.3. Collagen XVI – a binding partner of α1β1 integrin and α2β1 integrin

During embryonic development α1β1 integrin and α2β1 integrin are both widely expressed (Gardner et al., 1996; Wu and Santoro, 1994). *In vivo* α1β1 integrin expression is restricted to few cell types including capillary endothelial cells, fibroblasts and smooth muscle cells (Voigt et al., 1995). Integrin α2β1 is predominantly found in

highly differentiated cells such as placenta cells and chondrocytes. Immunohistochemical studies reveal its expression in fibroblasts, endothelial cells, on Schwann cells of ganglia and non neuroglia and epithelial cells from multiple sites (Wu and Santoro, 1994). A large variety of collagens from different families are able to act as ligands for integrins α1β1 and α2β1, including fibril forming collagens, FACIT collagens or basement membrane collagens (Table 2-1).

Both integrins α1β1 and α2β1 mediate cell adhesion, cell migration and contract three-dimensional collagen lattices. This contractile ability *in vitro* reflects their potential to modulate collagen-rich matrices. Analysis of the collagen-binding integrins on liver stellate cells reveals integrin α1β1 as the major integrin on myofibroblasts *in vivo* mediating collagen contraction while integrin α2β1 is induced upon *in vitro* culture. The ability of α1β1 integrin to mediate collagen gel contraction by stellate cells may take part in collagen turnover and collagen reorganization in liver fibrosis (Racine-Samson et al., 1997). Mice deficient of the α1β1 integrin are viable, however, they display a proliferation defect and a disturbed regulation of collagen synthesis in skin fibroblasts (Gardner et al., 1996; Gardner et al., 1999; Pozzi et al., 1998). Antibody studies and the use of integrin α1-chain-deficient mice have shown that integrin α1β1 plays an important role in inflammatory reactions and is a target in anti-fibrotic and anti-inflammatory treatments (Gullberg and Lundgren-Akerlund, 2002). Integrins α1β1 and α2β1 are mainly involved in tissue injury and tissue inflammatory situations (Gullberg and Lundgren-Akerlund, 2002).

The binding site of α1β1 integrin was localized to the C-terminal tryptic collagen XVI fragment comprising the collagenous domains COL1-3. α1β1 integrin requires the homotrimeric sequences GFOGER and/or GLOGER, however, does not tolerate the substitution of the arginine residues for a lysine residue (Xu et al., 2000). Collagen XVI contains related amino acid sequences GLQGER (1065-1070) and GIKGER (1101-1106) within the N-terminal part of the COL2 domain (Eble et al., 2006; Pan et al., 1992). α1β1 integrin binds most likely to either one or both of the collagenous integrin recognition sites in the COL2 domain. The binding of α2β1 integrin to collagen XVI is much weaker compared to α1β1, however, it binds to the same region without this binding site being mapped in detail. The best decribed high affinity ligand of α1β1 integrin is the heterotrimeric collagen IV, however collagen XVI binds with similar strength (Eble et al., 1993; Kern et al., 1993). The COL2 domain of collagen XVI harbours another integrin adhesion sequence, RGD. However, this binding site is not

accessible to RGD-dependent integrins within the triple-helical conformation (Eble et al., 2006). Additionally, the collagen-binding integrins α1β1 and α2β1 are independent of this RGD sequence (Eble et al., 1993; Eble et al., 2001). It has been proven that other putative binding sites like GGKGER (337-342) and GKAGER (672-677) located in the COL10 and COL6 domains of collagen XVI are no functional recognition sites for α1β1 integrin. For both integrins, it has been demonstrated that they interact with their A domain of the α-subunit with collagen XVI (Eble et al., 2006). This suggests similar binding mechanisms as to other collagens (Emsley et al., 2000).

2.4. Pathological disorders involving collagen XVI

2.4.1. Collagen XVI in tumorigenesis

The fibril forming collagens I, III, V and VI are usually not present in the brain parenchyma, but they are components of mesenchymal structures. In gliomas only few tumor cell types deposit collagens in the peritumoral zone. Collagen IV, a major component of basement membranes is deposited by endothelial cells and choroid plexus epithelial cells in healthy brain. In glioblastomas collagen IV is present in virtually all tumor vessels as well as in some giant glioma cells and in tumor cells around vascular proliferations. Some types of gliomas develop a collagen network around individual tumor cells. The expression pattern of collagens changes during the growth of gliomas in cell culture. A phenomenon known as mesenchymal shift manifests in deposition of collagens I, III, IV and VI by glioma cell lines (Paulus et al., 1994). Invasion of glioma cells into brain tissue is facilitated by remodelling of tumor associated ECM (Claes et al., 2007). By using differential gene expression microarray analysis to compare glioma cells with increased versus decreased migratory phenotype, an upregulation of collagen XVI gene expression in a subpopulation of cells showed increased migration (Tatenhorst et al., 2004). Collagen XVI has been the first member of the FACIT-collagens that was designated to tumorigenesis of glioblastomas. It could be detected in tumor vessels and tumor cells. Glioma tissue and a subset of glioma cell lines showed an upregulated expression and secretion of collagen XVI. Glioma cell lines adhered strongly to recombinant collagen XVI while this adhesion was dramatically reduced after blocking the endogenous collagen XVI expression. However, recombinant collagen XVI did not have any effect on migration properties of these cell lines (Senner et al., 2008). Low collagen XVI expression in the central nervous system of adult mice corroborates the absence of collagen XVI expression in normal human brain (Lai and

Chu, 1996). Neuronal cell movement is related to collagen XVI expression by neurite growth cones following axotomy (Hubert et al., 2007).

Collagen XVI expression in glioma cells is due to the mesenchymal drift of tumor cells during cell culture (Paulus et al., 1994), since tumor cells express collagen XVI in situ. Transforming growth factor-ß 2 (TGF-ß2) is upregulated in gliomas and induces collagen XVI expression in fibroblasts (Arslan et al., 2007; Grassel et al., 1998). Members of the FACIT collagens interconnect collagens and other components of the extracellular matrix and therefore collagen XVI may support the migration of glioma cells. Although, no migration promoting effect of recombinant collagen XVI in monolayer migration assays could be observed collagen XVI may act as migration promoting substance in combination with other factors. Collagen XVI supports several glioma cell lines in their adhesion (Senner et al., 2008). The integrin α1β1 is expressed on glioma cell surfaces and has been demonstrated to interact with high affinity with collagen XVI (Eble et al., 2006). This integrin is involved in cell adhesion and migration and controls tumor-ECM-interaction (Paulus et al., 1993). Knockdown of endogenous collagen XVI in the glioma cell line U87MG reduces this adhesion capacity. Glioma cells modulate their environment by modulation of ECM components resulting in altered adhesion and migration (Zamecnik, 2005). Collagen XVI fragments have been detected in glioblastoma tissue and glioma cells resembling the 180 kDa form lacking the N-terminal NC11 domain and the 130 kDa form which misses C-terminal regions (Kassner et al., 2004). Proteolytic fragments from other collagens (type IV, type XVIII) influence glioma cell growth and migration (Bix and Iozzo, 2005), therefore, also collagen XVI could act mitogenically rather by its fragments than by the full length protein. Gliomas secrete a large variety of matrix metalloproteinases (MMP-2, MMP-9) that could have generated the different collagen XVI fragments (Annabi et al., 2009; Nakada et al., 2003). It is speculated that the glioma itself regulates cellular functions by proteolytically cleaved collagen XVI fragments (Senner et al., 2008).

2.4.2. *Inflammatory bowel disease – Crohn's disease*

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are distinguished as clinical subtypes. In 1932, Crohn, Ginzburg and Oppenheimer described in their paper 'a disease of the terminal ileum, affecting mainly young adults and characterised by a subacute or chronic necrotizing and circatrizing inflammation. The ulceration of

the mucosa is accompanied by a disproportionate connective tissue reaction which frequently leads to stenosis of the lumen of the intestine associated with the formation of multiple fistulas.' During the following years the term Crohn's disease had been introduced. CD can affect any part of the intestinal tract and leads to discontinuous transmural lesions of the bowel wall by an inflammatory process with mucosal damage, the presence of non caseating granulomas and fissures with formation of fistulas. UC inflammation is limited to the colon and rectum with superficial but continuous lesions and affects only the mucosa and submucosa except for most severe cases. In contrast to CD, UC extends continuously and proximally from the rectum. Annually 6 to 10 incidences of CD per 100,000 occur in Western Europe and Northern America (Shivananda et al., 1996).

2.4.2.1.   The bowel wall – an introduction

The intestinal wall comprises the mucosa (epithelial cells, lamina propria, lamina muscularis mucosae), submucosa, muscularis propria and serosa (Fig. 2-4). The inner lumen of the bowel wall is separated by the epithelial cell layer immobilized on the basal lamina. The main part of the mucosa consists of the lamina propria containing lymphocytes, macrophages, fibroblasts and capillaries within the extracellular matrix. The lamina propria is followed by the lamina muscularis mucosae and the submucosa. Lamina muscularis mucosae comprises about five cell layers of smooth muscle cells and is essential in the intestinal motility, whereas the submucosa is characterized as a loose meshwork of thin collagen fibrils connecting mucosa and muscularis propria and guiding blood and lymphatic vessels. Densely packed smooth muscle cells form the muscularis propria which is surrounded by collagen fibrils. These fibrils are interconnected with intramuscular septae. The longitudinal muscle layer is defined by connective tissue, the serosa, which separates the bowel from the peritoneum by an epithelial cell layer.

epithelial cells

lamina propria

lamina muscularis mucosae

submucosa

circular muscle layer

longitudinal muscle layer
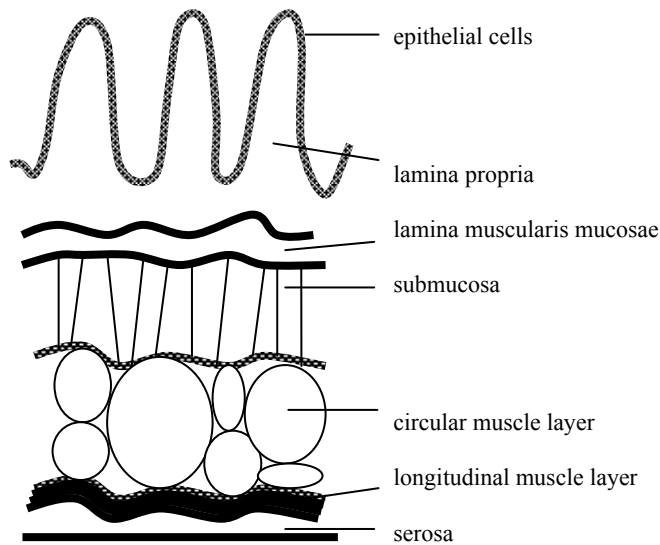
serosa

Figure 2-4: Composition of the normal bowel wall

The intestinal wall comprises the mucosa (epithelial cells, lamina propria, lamina muscularis mucosae), submucosa, muscularis propria, and serosa.

2.4.2.2.   Pathophysiology and molecular mechanisms of CD

The molecular basis of the pathogenesis of IBD is not yet clear, however, persistent bacterial infection, a defective mucosal barrier and an imbalance in the regulation of the intestinal immune response may contribute to the clinical manifestation of IBD (Podolsky, 2002). Chronic inflammation of the gastrointestinal tract is the main manifestation in CD and often accompanied by other systemic abnormalities. Terminal ileum and proximal colon are mainly affected by CD accompanied by an up-regulation of T-helper cells type 1 and pro-inflammatory cytokines like interferon-γ and interleukin-2. Monocytes and macrophages are stimulated to produce higher levels of TNF-α, interleukin-1 and interleukin-6. Individuals with a genetic susceptibility react to the bacterial flora in the intestinal tract with an abnormal inflammatory immune response (Shanahan, 2002). Established inflammatory lesions are regarded as the precondition for the onset of intestinal fibrotic processes. The excessive tissue repair is caused by a pathologically increased healing response to inflammation-induced disintegration of mucosal tissue. In contrast to normal wound healing chronic inflammation persists and occurs simultaneously with inflammation, tissue remodelling and repair processes (Wynn, 2007). Moreover, in fibrotic and wound granulation tissue extensive cell-matrix contacts occur (Singer et al., 1984).

2.4.2.3. Extracellular matrix composition in the colon

In the gut predominant connective tissue proteins are the ubiquitous interstitial collagens type I, II, III, type IV in basement membranes and type V as pericellular collagen. The luminal surface of the mucosa is lined by epithelial cells. This cell layer covers a continuous basement membrane consisting of type IV collagen, heparin sulphate proteoglycan and laminin (Stanley et al., 1982). The underlying lamina propria supports the epithelium and represents a loose network of connective tissue comprising collagen types I, III, IV and V. The lamina propria facilitates absorption and secretion through capillaries and lymphatics. A compilation of mesenchymal cells including smooth muscle cells, subepithelial myofibroblasts and fibroblasts contribute to the extracellular matrix deposition.

A thin layer of smooth muscle cells separates the mucosa from the submucosa and facilitates motility of the villi. The submucosa, a network of collagen fibrils connects the mucosa to the muscularis propria. The specific composition of collagen fibrils allows the mucosa to move over the muscularis propria (Graham et al., 1988; Komuro and Hashimoto, 1990). Densely packed smooth muscle cells, embedded in collagen fibrils represent the muscularis propria. In the human intestinal muscle predominantly collagen types I and III are present.

2.4.2.4. Intestinal wound healing and fibrosis

The architecture of the gastrointestinal tract is specified by a hollow, mucosa-lined muscular tube. Its functionality comprises an aboral movement of chyme, the absorption of water, electrolytes and nutrients and defense against harmful agents. The gut bears a huge capacity of healing itself after injury. Ineffective healing would result in necrotic tissue, perforation and the spillage of luminal contents into the peritoneum which may be lethal. Therefore, the healing function preponderates the organ function. Stricture formation is the natural response to chronic inflammation in CD, however, it may reduce its functionality (Graham et al., 1988).

2.4.2.5. The role of intestinal myofibroblasts

Mesenchymal cells like fibroblasts, myofibroblasts and smooth-muscle cells are the main producers of extracellular matrix components and play an important role in tissue growth and development in different tissues (Powell et al., 1999 ; Simon-Assmann et

al., 1995). Myofibroblasts are considered as central players in tissue repair contributing to fibrosis and stenosis by reconstituting collagen-rich ECM and promoting wound closure by contraction (Tomasek et al., 2002). Normal wound healing would terminate contractile and synthesizing activity of myofibroblasts by reduction of cell number through apoptosis (Desmouliere and Gabbiani, 1995). Chronic pathological tissue contractures, however, are characterized by continuity of exessive contractile and synthesizing activity over several years (Desmouliere et al., 2005 ; Gabbiani, 2003). It is an important prerequisite to understand how and why cells gradually loose their integrity in different phases of pathophysiological processes to improve diagnosis and therapy. The aim is to limit the progression of fibrosis as soon as possible without affecting the physiological repair process. A potential strategy to interrupt the impaired regeneration is to induce the disappearance of myofibroblasts from fibrotic tissue. Therefore, a modification of the regenerative potential involving matrix adhesion of myofibroblasts would be most effective (Desmouliere et al., 2005).

2.4.2.6.   Cell-matrix interaction in Crohn's disease

The delicate homoestasis between cells and surrounding ECM is already disturbed at the onset of CD and the levels of several fibrillar and non-fibrillar collagens (type I, III, IV, V, VI) are upregulated (Graham et al., 1988 ; Matthes et al., 1992 ; Pucilowska et al., 2000 ; Stallmach et al., 1992). Transmembrane receptors of the integrin family physically connect the ECM to the cellular cytoskeleton relaying to multiple signaling pathways. Thus ECM components and intracellular stress fibers are linked by actin coupled cell-matrix adhesions. This association with intracytoplasmic actin filaments enables force transmission from the myofibroblast to the substrate while intercellular connections synchronize myofibroblast contraction. Actin-integrin adhesion complexes involve several signaling pathways which in turn modulate complex formation (Geiger and Bershadsky, 2001). Initial ligation is followed by integrin attachment to the actin cytoskeleton (Jiang et al., 2003) and tractional forces are transmitted via adhesive structures called focal adhesions (FA) or focal contacts (FC) (Harris et al., 1980 ; Pelham, Jr and Wang, 1999). Usually, apoptotic processes occur after reconstitution of physiologically healing wounds and the myofibroblasts disappear (Desmouliere et al., 2005), however, in fibrocontractive and –proliferative diseases the contractile activity of cells persists and leads to a continous remodelling and retraction of the ECM.

2.4.2.7.   Treatment of Crohn's disease

Treatment of the disease either by drugs or surgical removal of inflamed intestinal parts do not result in complete cure of CD. However, there are several treatments available to allay the symptoms. Aminosalicylates, glucocorticoids, antibiotics, immunosuppressives and monoclonal antibodies are applied in different stages and localization of the disease according to table 2-2.

Disease activity	Symptoms	Medication	Alternatives
Low	Inflammation of the terminal ileum and/or colon	Glucocorticoids	No antibiotics
Moderate	Inflammation of the terminal ileum and/or colon	Glucocorticoids, additional antibiotics	
Acute	Inflammation of the ileum and/or colon	Systemic glucocorticoids, if recidivism also immunosuppressives	If therapy is not successful: use of monoclonal antibodies
Highly acute	Large inflammation of the bowel	Systemic glucocorticoids, aminosalicylates,	If therapy is not successful: use of monoclonal antibodies

Table 2-2: Indications for the medication of Crohn's disease according to the guidelines of the European Crohn's and Colitis Organization (ECCO).

2.5. Gene-function analysis – RNA interference as a method of choice

RNA interference (RNAi) is a potent tool to study the effects of the loss of a single protein. It is a valuable alternative to knock-down models in mice or zebrafish. Double-stranded RNAs (dsRNA) are cleaved in 21-25 nucleotide (nt) short small interfering RNAs (siRNA) (Elbashir et al., 2001). These in turn initiate sequence-specific degradation of mRNA by simulation of endogenous microRNA (miRNA). Endogenous and exogenous interfering RNA duplexes are incorporated with a RNA-induced

silencing complex (RISC) which attaches to complementary mRNA resulting in gene silencing (McManus and Sharp, 2002).

2.5.1. Gene suppression by short interfering RNA

The central molecule in RNAi is dsRNA which is initially cleaved into short RNA fragments of around 23 bp by the Dicer molecule (Bernstein et al., 2001). Dicer starts at one end of the dsRNA and ATP-dependently cleaves fragments of 21-23 bp from the dsRNA. The siRNA is incorporated into a protein complex named RISC (RNA-induced silencing complex) (Hammond et al., 2000). RISC is activated by the ATP-dependent unwinding of the siRNA duplex. The active RISC recognizes a homologous target mRNA by base pairing and subsequently cleaves the mRNA at approximately 12 nt from the 3'-end of the siRNA. The siRNA strand is removed and most likely degraded, (Sijen et al., 2001; Smardon et al., 2000) and the resulting dsRNA again enters the RNAi circuit. On the other hand the nicked mRNA is further degraded and the RISC complex may be recycled to perform additional mRNA degradation.

Establishing RNAi in mammalian cells is complicated by non-specific gene silencing and by profound physiological responses to dsRNA often leading to cell death. Here, the length of siRNA is essential. One single molecule of dsRNA longer than 30 nucleotides is sufficient to trigger the interferon response. This anti-viral response results in global inhibition of translation (Manche et al., 1992) and mRNA is unspecifically degraded (Minks et al., 1979), finally leading to apoptosis (Clemens and Elia, 1997; Gil and Esteban, 2000; Sekellick and Marcus, 1985).

2.5.2. shRNA-mediated silencing of mammalian gene expression

The simplest secondary RNA structure is the hairpin structure. It is formed by a single-stranded RNA folding back onto itself, resulting in a double-stranded RNA stem joined at one end by a single-stranded loop. The role of the hairpin RNAs is defined by tertiary interactions in RNA enzymes, protection of mRNAs from degradation and recognition by RNA-binding proteins. The sequence of the loop is almost unimportant as long as one stem strand is complementary to the targeted sense mRNA sequence (Brummelkamp et al., 2002; Paddison et al., 2002). Chemically synthesized DNA oligonucleotides that encode the chosen sequence are annealed to a double-stranded fragment. This fragment is subsequently cloned into a hairpin expression vector downstream from an eukaryotic promoter. DNA sequencing has to be performed since

oligonucleotides that encode hairpins are relatively long (60-90 nucleotides) and possess a high degree of secondary structure. The introduction of shRNA constructs to cells can be accomplished by using any gene transfer technique, however, systems based on retroviral, lentiviral or adenoviral expression guarantee a well-characterized stable expression of shRNAs in target cells (Castanotto et al., 2002). For the gene silencing of collagen XVI we use a retroviral mediated mechanism. The retroviral expression vector is inserted into a packaging cell line. This cell type already contains genes for virus particle formation and replication, stably integrated into the genome. The retroviral expression vector provides the packaging signal ψ^+, transcription and processing elements, and the target gene which are resulting in production of a replication-incompetent virus. A viral *env* gene encodes the envelope protein, which determines the range of infectivity (tropism) of the packed virus. The utilized ecotropic virus can only recognize a receptor expressed on mouse and rat cells. An antibiotic selection can be used to select cells that stably express the integrated vector. The virus produced by transfections can infect target cells and transmit genes of interest, however, it cannot replicate within the target cell. The whole mechanism is depicted in Fig. 2-5.
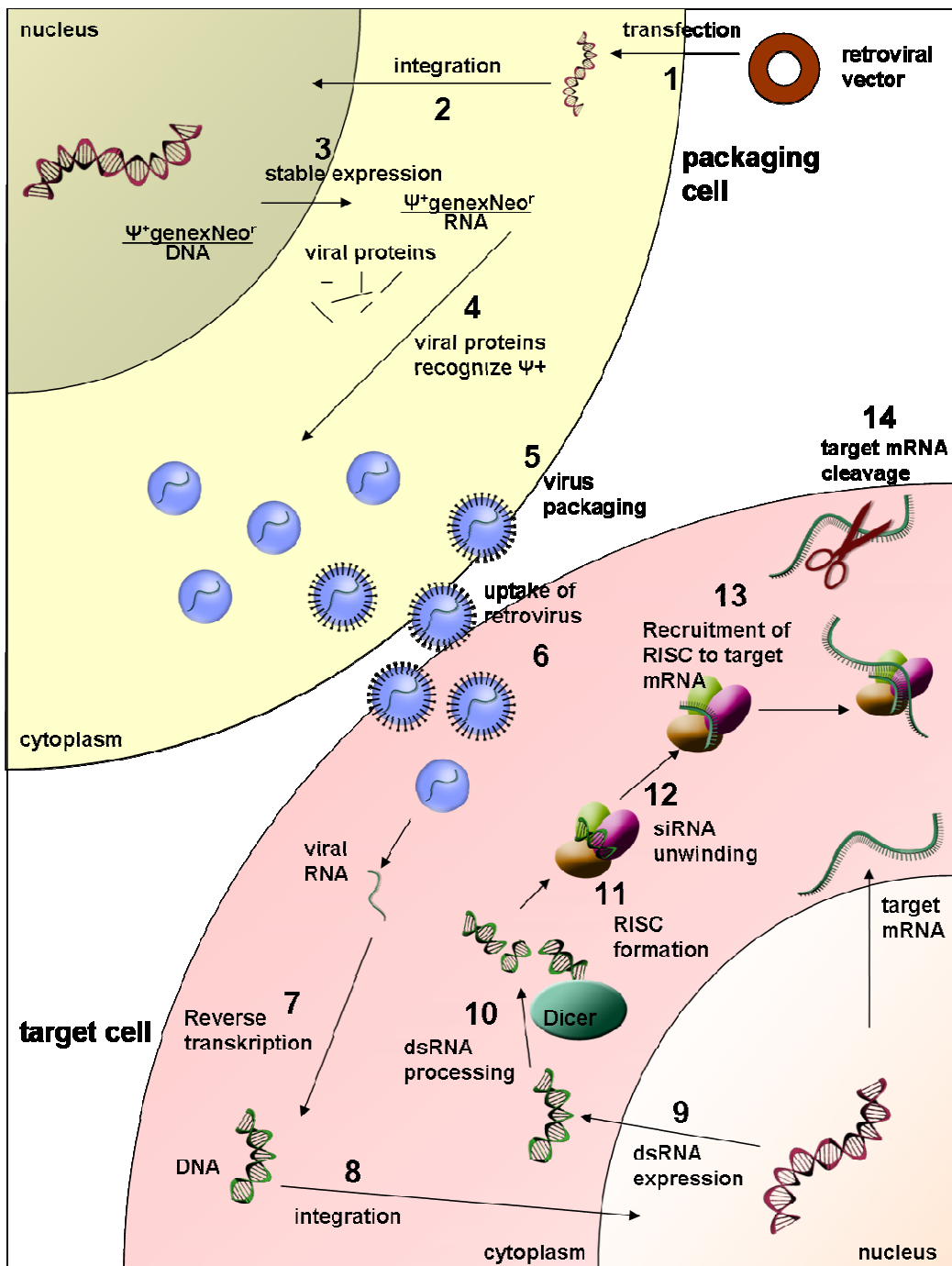
Figure 2-5: Mechanism of retroviral mediated gene silencing. The retroviral expression vector contains a puromycin resistance gene for the selection of stable transfectants and is transfected (1) into the cell and stably integrated into the genome of the packaging cell line (2). The packaging signal ψ^+, transcription and processing elements, the target gene and viral proteins are stably expressed by the packaging cell line (3). The viral proteins recognize the packaging signal (4) and the virus is packed and transported

outside the cell (5). The target cell can be infected by uptake of the retrovirus (6). The viral RNA is transcribed into DNA (7) and stably integrated into the genome of the target cell (8). dsRNA is expressed (9) and processed by Dicer into siRNAs (10). The RISC complex is formed with siRNAs (11), the dsRNA is unwound (12) and results in an active RISC complex. The complex is recruited to the target mRNA (13) and finally leads to a specific mRNA cleavage (14).

3. Aim of the thesis

The highest reward for man's toil is not what he gets for it, but what he becomes by it. – John Ruskin

Background

The gene and protein sequence of collagen XVI was described in 1992 (Pan et al., 1992). Since then, it has been structurally and ultra-structurally characterized including post-translational modifications, possible cleavage- and integrin binding sites. Collagen XVI has been investigated in skin, cartilage and neural tissue. In the human skin it is part of the dermo-epidermal junction (DEJ) associated to the microfibrillar apparatus which is formed by fibrillin-1 containing microfibrils (Grassel et al., 1999). It has also been detected close to the basal membrane and in smooth muscle cells derived from blood vessels. In cartilage it is present in the territorial and pericellular zone of chondrocytes, where it is associated to the surface of D-periodically banded cartilage fibrils (Kassner et al., 2003). However, in cartilage it does not interact with fibrillin-1 as in skin. The suprastructurally different appearance of collagen XVI also indicates a tissue-specific functional diversity. For skin an interaction and connection of the microfibrillar apparatus with structures of the basal membrane via collagen XVI is proposed, whereas in cartilage collagen XVI seems to modify the fibrillar surface and to modulate interfibrillar interactions.

The role of collagen XVI in neural tissue has also been the focus of several investigations. Collagen XVI is highly expressed in the peripheral nervous system during embryonal development and is re-expressed after axotomy. This specific expression pattern indicates that collagen XVI is involved in regeneration processes (Hubert et al., 2007). During progression of tumorigenesis of glioblastoma and astrocytoma collagen XVI was highly up-regulated. This suggests a role of collagen XVI in the course of the integration of tumors into the surrounding tissue (Senner et al., 2008) where collagen XVI might support tumor integration into the healthy tissue.

Collagen XVI and Crohn's disease

Expression pattern of collagen XVI in healthy and diseased tissue

Crohn's disease is a heterogenous entity which comprises several disease presentations: fibrotic strictures, penetration and chronic inflammation. As collagen XVI is up-regulated in fibrotic diseases of the skin, we propose a role for this collagen in intestinal fibrotic diseases like Crohn's disease. Collagen XVI is present in the intestinal system

and its molecular distribution and deposition pattern in the human physiological and pathological situation is one important investigation target. Here, immunofluorescence staining of collagen XVI in healthy and diseased tissue from CD patients is applied.

The influence of the inflammatory progression of Crohn's disease on collagen XVI expression and deposition

CD patients progress through different stages of inflammation during the course of disease. Several collagens (collagen type I, III, IV, VI) have been reported to be up-regulated in CD, however, irrespective to the grade of inflammation. We propose a role for collagen XVI in cell-cell and cell-matrix interactions dependent on inflammatory progression. Subepithelial myofibroblasts are central players in extracellular matrix deposition in the healthy intestines and in intestinal fibrotic diseases. The gene and protein expression of collagen XVI in these cells was investigated in immunofluorescence staining, immunoblotting and quantitative PCR.

Characterization of intestinal subepithelial myofibroblasts and their interaction with collagen XVI

Intestinal subepithelial myofibroblasts are important in the extracellular homeostasis, however, still not well described. Another aim of this work is to characterize these cells in their adhesion, proliferation, and migration properties with respect to their interaction with collagen XVI. Underlying mechanisms of cell-matrix interactions and integrin mediated processes involving collagen XVI are another investigation target. Spreading and attachment of the cells on several substrates (poly-L-lysine, 5 µg/ml; collagen I, 10 µg/ml, collagen XVI, 10 and 30 µg/ml) is determined employing morphometric analysis of focal adhesion contacts and integrin expression pattern is investigated by flow cytometry and immunofluorescence. The same substrates are applied to analyze their influence on proliferation and migration capacity.

Functional analysis of collagen XVI based on a gene silencing model
Establishment of a stable knockdown of collagen XVI

The reason of structural diversity of collagen XVI is still unclear. An approved method to determine the function of a protein is to deplete it as much as possible. In absence of a valid knock-out mouse model and in order to have a reproducible *in vitro* tool, RNA interference is the matter of choice. For this work a retroviral gene delivery system is established to obtain a knock-down of collagen XVI in dividing murine cells.

Analysis of the effects of the knockdown of collagen XVI in NIH3T3 fibroblasts

With 2D-gelelectorphoresis differential expression of other proteins in the presence and absence of collagen XVI are analyzed, further identified with mass spectrometry, and approved in gene and protein expression by quantitative PCR and immunoblotting.

Differences in cell proliferation and adhesion of NIH3T3 fibroblasts are determined by appropriate assays.

Summary

Aim of this work is to elucidate the role of collagen XVI during the progression of CD and to identify cellular mechanisms of ISEMF involving collagen XVI.

Additionally, a gene-function analysis of collagen XVI is performed by establishment of a retroviral mediated knockdown model in NIH3T3 fibroblasts.

4. Material and methods

The true method of knowledge is experiment. – William Blake

4.1. Microbiological methods

4.1.1. Cloning with TOPO-TA vector systems

Topo TA cloning has been used for efficient cloning of PCR products with a Taq polymerase that has a non-template-dependent terminal transferase activity.

Reagent	Volume
Fresh PCR product	0.5-4 µl
Salt solution	1 µl
Water; add to a total volume of 5 µl	
TOPO® vector	1 µl
Final volume	5 µl

Table 4-1: Ligation set up.

The ligation set up (Table 4-1) was incubated at room temperature for 5 min and finally transformed into competent TOP 10 E. coli by heat shock as described in 4.1.4.

4.1.2. Vector constructs for retroviral gene silencing using RNAi-ready pSIREN Retro Q

Three individual shRNAs for collagen XVI were selected applying two algorithms provided by Clonetech, BD Biosciences and Invitrogen (Table 4-2). The hairpin loop containing shRNAs were generated and purchased from MWG, Germany.

<u>shRNA 1</u>
Start: 326
Target sequence gcaagaaggactcaagcta

Top strand
5'gatcc gcaagaaggactcaagctattcaagagatagcttgagtccttcttgcttttttacgcgt g---- 3'

Bottom strand
5'aattc acgcgtaaaaaagcaagaaggactcaagctatctcttgaatagcttgagtccttcttgc g---- 3'

<u>shRNA 2</u>
Start: 571
Target sequence ggaacacatggtacttgtt

Top strand
5'gatcc ggaacacatggtacttgttttcaagagaaacaagtaccatgtgttcctttttttacgcgt g---- 3'

Bottom strand
5'attc acgcgtaaaaaaggaacacatggtacttgtttctcttgaaaacaagtaccatgtgttcc g---- 3'

<u>shRNA 3</u>
Start: 2059
Target sequence agggtagttttggcgatac

Top strand
5'gatcc aggtagttttggcgatacttcaagagagtatcgccaaaactacccttttttttacgcgt g---- 3'

Bottom strand
5'aattc acgcgtaaaaaaagggtagttttggcgatactctcttgaagtatcgccaaaactaccct g---- 3'

Table 4-2: shRNAs for *collagen XVI* silencing.

4.1.3. Bacterial culture medium

Culture medium	Concentrations	Reagents
LB-medium (pH 7.5)	10 g/l	Bacto Trypton-extract (BD, Germany)
	5 g/l	Bacto yeast-extract (BD, Germany)
	85 mM	NaCl (AppliChem, Germany)
SOC-medium (pH 7.5)		Obtained from Invitrogen, Germany
LB-agar	1.5 % (w/v)	LB-medium containing Bacto Agar (BD, Germany)

Table 4-3: Bacterial culture medium components.

LB-medium was autoclaved and cooled below 50 °C before the antibiotic was added. LB-agar was prepared similarly by autoclaving LB-medium which contains appropriate amounts of powdered agar. Antibiotic was added prior to preparing agar plates under sterile conditions (Table 4-4). The culture plates were stored at 4 °C.

Antibiotic	Final concentration
Ampicillin, Sigma, USA	100 µg/ml
Kanamycin, Sigma, USA	25 µg/ml

Table 4-4: Antibiotics for bacterial selection medium.

4.1.4. Transformation of competent bacteria and cloning of cDNA fragments

4.1.4.1. General transformation protocol

Commercially available competent bacteria E. coli XL GOLD and TOP 10 were obtained from Stratagene (Netherlands) and Invitrogen (Germany), respectively. The bacteria were transformed and transformed colonies and bacteria stocks were stored in cryotubes at -80 °C (roti®store, Roth, Germany).

Transformation was performed employing the heat shock treatment method. For this purpose 25 µl of bacterial suspension was mixed with the DNA solution and incubated on ice for 30 min. This mixture was placed into a water bath at 42 °C for 45 sec and immediately cooled on ice. The bacterial suspension was mixed with 500 µl SOC-medium and placed at 37 °C with shaking (200 rpm) for 45 min. Aliquots of the suspension (50 µl and 100 µl) were plated on LB agar plates containing appropriate antibiotics and were incubated over night at 37 °C. Plates containing bacterial colonies were either stored at 4 °C until further use or single colonies were transferred to 5 ml antibiotic containing medium and incubated over night at 37 °C, 200 rpm shaking. The correct insertion of the DNA-fragment was approved by colony PCR as described in 4.2.2, by final plasmid isolation (Qiagen Spin Miniprep Kit, according to the manufacturer's protocol) and sequencing of the insert (performed at Geneart, Germany).

4.1.4.2. Transformation with recombinant pSIREN-RetroQ Retroviral vector

After ligation of the shRNAs into the linearized shRNA expression vector (RNAi-ready pSIREN retro Q plasmid expression vector system, Clonetech, BD, Bioscience) this vector system was transformed into 50 µl of E.coli K-12 competent cells (Fusion-Blue

competent cells, Clonetech, USA). After 30 min incubation on ice the cells were subjected to a heat shock for 45 sec at 42 °C in a water bath and placed back on ice. Transformation was performed at 37 °C for 1 hr shaking at 200 rpm after the addition of 950 µl SOC medium. 50 µl of the transformation mix were plated on LB agar plates containing ampicillin (see antibiotics 4.1.3) and incubated overnight at 37 °C.

4.2. Molecular biology methods

4.2.1. Isolation and purification of plasmid DNA

Plasmid DNA was isolated by using a silica membrane system commercially available from Qiagen, Germany. According to the manufacturer's protocol for Spin Miniprep kits 5 ml of bacteria suspension were initially applied. Higher volumes up to 50 ml were purified with Qiagen Midiprep kit yielding up to 500 µg plasmid DNA. Plasmids for retroviral gene silencing were isolated by an endotoxin free plasmid extraction kit (NucleoBond Plasmid Maxi EF kit, Clonetech, BD Bioscience, USA) from an overnight culture of bacterial colonies in 4 ml of liquid LB medium which contained ampicillin. Positive clones were identified by restriction- and sequence analysis.

4.2.2. Qualitative end-point Polymerase-Chain-Reaction (PCR)

The amplification protocol comprised denaturation of the cDNA template at 95 °C for 30 sec into single stranded DNA, specific annealing at 60 °C for 30 sec, and elongation at 72 °C for 10 min. This cycle was repeated 30 – 35 times to obtain higher concentrations of the original cDNA template. The table below displays a set up for a 50 µl PCR reaction using Hot Start Taq polymerase from Invitrogen, UK. Transformations were approved by colony PCR. In this case cDNA template was replaced by 1 µl bacterial suspension of an overnight culture.

Component	Concentration / volume
cDNA template	10-100 ng / 1-20 µl
10x PCR buffer	5 µl
10 mM dNTP Mix	1 µl
Primers (10 µM)	1 µl (each)
Hot Start Taq polymerase	0.25 µl
Water	Up to 50 µl
Total volume	50 µl

Table 4-5: PCR set up.

4.2.3. *Quantitative real-time Polymerase-Chain-Reaction (qPCR)*

Absolute and relative quantitative qPCRs were performed with SYBR Green Dye I with an ABI 7000 Prism Sequence detection system (Applied Biosystem, USA). Each primer was suspended in a concentration of 0.2 µM in SYBR Green master mix (Invitrogen, UK) and the reaction was carried out in 96-well plates with 5 µl diluted cDNA in a final volume of 25 µl. All primer pairs were run with the same amplification parameters which were repeated for 40 cycles. Denaturation took place at 95 °C for 9 sec and annealing at 60 °C for 1 min. Experiments were performed in triplicates for each sample. Murine and human primers were designed with Primer3 software and obtained from MWG-Biotech as listed in table 4-6 and table 4-7.

4.2.3.1. Relative quantification

Relative quantification was determined by the ΔΔCt method using an internal calibrator and GAPDH or 18sRNA as endogenous control. The mean relative quantification (RQ) values are plotted semi-logarithmically. RQ values were calculated using the RQ study application v1.1 software (ABI Prism 7000 SDS software v1.1).

4.2.3.2. Absolute quantification

For absolute quantification a standard curve of serially diluted plasmids of known copy number (250 up to 5×10^7) was plotted and sample Ct values were used to determine the exact copy numbers of the genes of interest.

Primer ID	Gene	RefSeq number	Amplicon (bp)	Sequence 5'-3'
mColXVIa_FW_4533	Col XVI	NM_028266	181	tacctccaggatgcagttcc
mColXVIa_RV_4714				cacctttggtaccaggcagt
mColXVIc_FW_559	Col XVI	NM_028266	248	aaacacaccttccggaacac
mColXVIc_RV_807				ctgggaggaagctgagacac
m18sRNA_FW_448	18sRNA	NR_03278	154	aaacggctaccacatccaag
m18sRNA_RV_602				cctccaatggatcctcgtta
mPpia_FW_313	PPIA	NM_008907	126	agcatacaggtcctggcatc
mPpia_RV_439				ttcaccttcccaaagaccac
mMIF_FW_224	MIF	NM_010798	106	atgacttttagcggcacgaa
mMIF_RV_330				aggccacacagcagcttact

Table 4-6: Murine oligonucleotides

Primer ID	Gene	RefSeq number	Amplicon (bp)	Sequence 5'-3'
hColXVI_FW_4689	Col XVI	M92642	97	gcctggtaccaaaggtgaaa
hColXVI_RV_4786				catagcctggaggaccttga
hCol_I_FW_2867	Col I	NM_000088	172	acgtcctggtgaagttggtc
hCol_I_RV_3038				accagggaagcctctctctc
hGAPDH_2F_4531	GAPDH	J04038	120	ctgacttcaacagcgacacc
hGAPDH_2R_4650				ccctgttgctgtagccaaat
hFN_FW_4326	Fibronectin	BC117176	128	cccaactggcattgactttt
hFN_RV_4453				ctcgaggtctcccactgaag

Table 4-7: Human oligonucleotides

4.2.4. Approvement of correct insertion of cDNA fragments

The plasmid cDNA inserts were characterized by isolating the cDNA fragment with restriction enzymes and application to an agarose gel containing ethidiumbromide to visualize the fragments. At least 200 ng of DNA were digested with 2 to 5 U of specific restriction enzymes in an appropriate buffer system for 2 hrs at 37 °C.

Correct insertion of shRNA fragments was approved first by digestion with the enzyme MLU 1 which resulted in cleavage within the shRNA insert and second by sequence analysis.

All restriction enzymes were purchased from NEB, UK.

4.2.5. Agarose gel electrophoresis

cDNA suspended in a loading buffer (6x agarose gel loading dye, Fermentas, Germany) was applied to agarose gels with varying agarose concentration from 1 % to 1.5 % (w/v) (Seakem Agarose, Biozym, Germany) in TAE buffer (0.8 mM Tris-Base, AppliChem, Germany; 0.8 mM acetic acid, Merck, Germany; 0.04 mM EDTA, AppliChem, Germany, pH 7.8) containing at least 20 mg/ml ethidiumbromide (Sigma, USA). An increasing concentration of agrose was referring to a smaller pore size and vice versa. Therefore, an electrophoretical size discrimination of cDNA amplicons was enabled. Comparison with a prestained marker (Gene Ruler, Fermentas, Germany) allowed control of the fragment size. The electrophoresis was perfomed at max. 90 V for 45 to 90 min. Gels were visualized in a Chemi-Imager (Intas UV systems, Germany).

4.2.6. Isolation, purification and quantitation of cDNA fragments

cDNA fragments were isolated from agarose gels with Qiagen gel extraction kits (Qiaquick, QIAEX II, Qiagen, Germany) according to the manufacturer's protocol. In order to remove nucleotides, enzymes or interfering salt concentrations the cDNA was purified using a PCR purification kit from Qiagen. Concentration of cDNA was determined by using an Eppendorf Biophotometer and evaluation of the absorption at 260 nm / 280 nm. Absorption of 320 nm was used to determine protein contamination of the sample. An absorption of 1.0 at 260 nm refered to 50 μg/ml double stranded DNA. The ratio of absorption values of 260 nm to 280 nm of about 1.7 to 1.9 was considered without contamination protein residues.

4.2.7.   Ligation and dephosphorylation of cDNA fragments

cDNA fragments were inserted into cloning vectors applying T4 ligase (NEB, UK) at 16 °C over night. cDNA and vector were usually mixed in molar relations of 1:1 up to 5:1 using 2.5 U T4 ligase with appropriate buffers in a 20 µl set-up.

The ligation procedure is often disturbed by insert-oligomer formation or re-ligation of the linearized vector ends. This process can be minimized by incubation with alkaline phosphatase (CIP, calf intestine phosphatase, Roche, Germany) which digests the 5'-phosphate ends and therefore inhibits a re-ligation by T4 ligase.

4.2.8.   Ligation of hairpin structures (shRNA) for retroviral gene silencing

To obtain the hairpin structure 100 µM of top and bottom strands, shRNAs were mixed equally and incubated for 30 sec at 95 °C and were annealed for 2 min at sequential temperatures of 72 °C, 37 °C, and 25 °C. 0.5 µM of annealed strands of each shRNA were ligated separately into 25 ng/µl linearized shRNA expression vector (RNAi-ready pSIREN retro Q plasmid expression vector system, Clonetech, BD, Bioscience, Fig. 4-3) using 400 U/µl T4 DNA ligase in the presence of 1.5 µl of 10x T4 DNA ligase buffer in a total reaction volume of 15 µl. After 3 hrs incubation at room temperature 2 µl of the ligation products were transformed into 50 µl of E.coli K-12 competent cells (Fusion-Blue competent cells, Clonetech, USA).

4.2.9.   RNA isolation

RNA was isolated with Qiagen Rneasy Mini Kit, based on affinity columns from either freshly isolated or frozen (-80 °C) cells according to the manufacturer's protocol.

RNA content and purity were determined at 260 nm/280 nm. An adsorption value at 260 nm of 1.0 refered to 40 µg/ml single stranded RNA. The ratio of adsorption values of 260 nm to 280 nm was optimal at about 1.8 to 2.0 and guaranteed pure RNA.

4.2.10. Reverse transcription

Up to 5 µg RNA was conversed to cDNA using 500 ng/ml oligo dT primers and 10 mM dNTPs which were heated to 65 °C and quickly chilled on ice. First strand buffer, 0.1 M DTT, and RNAse OUT (40 U/rct) were mixed and added to the RNA-mixture and incubated for 2 min at 42 °C. First strand was synthesized after the addition of 200 U of SuperScript II enzyme (Invitrogen, UK) for 50 min at 42 °C. The reaction was

inactivated for 15 min at 70 °C and the cDNA was stored in aliquots until further use in polymerase chain reaction at -20 °C.

4.3. Protein biochemistry

4.3.1. Protein quantification

Protein content was determined using the BCA method according to Smith et al. 1985. This quantification method is based on the chemical biuret reaction. Proteins reduce Cu^{2+}-ions to Cu^+-ions under alkaline conditions. Bicinochoninacid (BCA; 2, 2'-Bichinolin-4, 4'-dicarbonacid-disodiumsalt, Pierce, USA) acts in a Cu^+ complexion. The purple complex adsorbs at 562 nm and detection of this adsorption maximum is related to protein concentration.

10 µl of the sample and a BSA standard solution (0 – 250 µg/ml in water) were separately added to 200 µl of BCA reagent. The final mixture was incubated for 30 min at 60 °C in a waterbath. The diluent of the sample served as blank. The extinction of the samples was determined in a photometer (Biophotometer, Eppendorf, Germany) at 560 nm and converted via linear regression of the BSA standard curve to protein concentration in ng/µl.

4.3.2. Protein precipitation

4.3.2.1. TCA precipitation

In order to concentrate proteins, the solution was incubated on ice for 20 min with a final concentration of 0.14 % (w/v) Triton X-100 (Sigma, USA) and 15.5 % (w/v) trichloric acid (TCA, Roth, Germany). Centrifugation with 18,000 x g for 20 min at 4 °C resulted in a protein pellet that was washed two times with pre-chilled acetone (Fischar, Germany). After air-drying the pellet was resuspended in an appropriate volume of sample buffer and protein content was quantified.

4.3.2.2. Methanol-chlorophorm precipitation

500 µl of protein sample were mixed with equal volumes of methanol and 0.4 x volumes of chloroform. This solution was thoroughly vortexed and centrifuged for 10 min at 15,000 x g. The mixture was separated into an upper aqueous and a lower organic phase divided by an interface containing the protein. The upper phase was removed and the residual sample was washed twice with 500 µl methanol and

centrifuged. After air-drying, the pellet was resuspended in an appropriate volume of sample buffer and protein concentration was determined with the BCA method.

4.3.3. SDS PAGE according to Lämmli

The discontinuous **s**odium-**d**odecyl-**s**ulfate **p**oly**a**crylamid-**ge**lelectrophoresis (SDS-PAGE) according to Lämmli, 1970 was used to discriminate heterogenous protein samples comprising different protein fractions. The proteins were separated for immunoblotting of collagen XVI in discrete gradient gels with a polymer content of 4.5 % up to 15 %. For 2D-gelelectrophoresis gels an acrylamide concentration of 10 % was used. For other applications polymer content was adapted according to the molecular weight of the sample protein. In all cases a 4.5 % stacking gel was poured on top of the resolving gel. Concentrations of the ingredients are listed in table 4-8. The gel run was performed under cool conditions at approximately 4 °C at a maximum current of 20 mA. A pre-stained protein marker was always co-separated to determine the molecular weight of the sample (Precision Plus™ Protein All Blue Standard, Biorad).

Solutions	Concentration of ingredients
Resolving gel buffer	1.5 M Tris/HCl, AppliChem, Germany pH 8.8 0,4 % (w/v) SDS, Roth, Germany
Stacking gel buffer	0.5M Tris/HCl, AppliChem, Germany pH 6.8 0.4 % (w/v) SDS, Roth, Germany
Acrylamide stock solution	30 % (w/v) bisacrylamide, Roth, Germany
TEMED	100 % triethylmethylethylendiamin, AppliChem, Germany
APS	10 % (w/v) ammoniumpersulfate (APS, Serva, Germany) in water
Sample buffer	0.1 M Tris/HCl, AppliChem, Germany, pH 6.8 1 % (w/v) SDS, Roth, Germany 20 % (v/v) glycerol, AppliChem, Germany 5 % (v/v) ß-mercaptoethanol, Sigma, USA 0.01 % (w/v) bromphenol blue, Sigma, USA

Table 4-8: Buffer concentrations for SDS-PAGE according to Lämmli.

4.3.3.1. Sample preparation for SDS PAGE

The protein sample had to be exempted from interfering ions and was therefore precipitated and spiked with sample loading buffer. The reduction of disulfide bonds was obtained by heating the samples with ß-mercaptoethanol in a final concentration of 5 % (v/v) for 5 min at 95 °C.

4.3.3.2. Protein detection in SDS PAGE

Proteins were stained after electrophoretic separation either with Coomassie Blue or silver nitrate or were prepared for blotting on a nitrocellulose membrane for immunodetection.

4.3.3.2.1. Coomassie Blue staining

The staining solution contained 0.1 % (w/v) Coomassie Brilliant Blue R-250 (Serva, Germany) in 25 % (v/v) methanol (Roth, Germany) and 10 % (v/v) acetic acid (Merck, Germany). Background staining was removed sequentially by two destaining solutions containing first 50 % (v/v) methanol, 10 % (v/v) acetic acid and second 10 % (v/v) methanol and 10 % (v/v) acetic acid, respectively.

4.3.3.2.2. Silver staining

The silver staining of polyacrylamid gels was perfomed using the following staining solutions listed in table 4-9.

Solutions	Concentrations of ingredients
Fixing solution	6 mM sodium thiosulfate ($Na_2S_2O_3$ x $5H_2O$), AppliChem, Germany 30 % (v/v) ethanol , Roth, Germany 400 mM sodium acetate, AppliChem, Germany 200 µl acetic acid, Merck, Germany 0.25 % (v/v) glutardialdehyde, Merck, Germany
Silver nitrate solution	6 mM silver nitrate, Fluka, Germany 0.019 % (v/v) formaldehyde, AppliChem, Germany
Developing solution	2.5 % (w/v) sodium carbonate, AppliChem, Germany

	0.015 % (v/v) formaldehyde, AppliChem, Germany
Stop solution	7 % (v/v) acetic acid, Merck, Germany

Table 4-9: Staining solutions for silver staining of polyacrylamide gels

The gels were fixed for 15 min in fixing solution on a shaker at 50 rpm. After several washing steps with distilled water the silver nitrate solution was allowed to incubate for additional 15 min. The protein bands were finally visualized with alkaline developing solution that reduced silver ions. The intensity of the staining was controlled by a stop solution.

4.3.4. Protein transfer for immunoblotting

Proteins were electrotransferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Germany) with a semi-dry blotter (MultiBlot-transfer, MBT, Germany) in a sandwich of three layers whatman paper (Schleicher & Schuell, Germany), nitrocellulose membrane, polyacrylamide gel and again three layers of whatman paper. Per cm² of nitrocellulose membrane 0.8 mA current was applied for 60 min up to 75 min according to the fragment size of the protein. Prior to transfer, Whatman paper and nitrocellulose membrane were soaked in blotting buffer containing 50 mM Tris (AppliChem, Germany), 380 mM glycine (AppliChem, Germany), 0.1 % (w/v) SDS (Roth, Germany), 10 % (v/v) methanol (Merck, Germany). After blotting, transfer of proteins was confirmed by a reversible Ponceau red staining of the membrane with a 0.2 % (w/v) Ponceau S solution in 3 % (v/v) TCA (both Sigma, Germany).

4.3.5. Immunoblotting (Western Blotting)

After protein transfer to a nitrocellulose membrane unspecific binding sites were blocked with a buffer containing 5 % (w/v) dry milk powder (Roth, Germany) in PBST (0.1 % (v/v) Tween 20 in PBS) for 1.5 hrs at rt. After a short washing step in PBST the membrane was incubated either at 4 °C over night or at rt for 2 hrs with the primary antibody diluted in blocking solution. Unspecific bound antibody was removed in three washing steps each for 10 min in PBST. The appropriate peroxidase coupled secondary antibody was also incubated in blocking solution for 90 min at rt. Washing steps were repeated as described for the primary antibody. Protein bands were detected by

incubating the membrane with ECL reagent (Pierce, USA) for 1 min. Wrapped in clear foil, the luminescent signal was detected by application of an x-ray film for 10 sec to 5 min (Amersham, UK). If StrepII-Tag was to be detected, dry milk powder was replaced by the same amounts of bovine serum albumin (BSA, Biomol, Germany) for blocking and all following incubation steps. This StrepTag interacts with high levels of biotin in milk and therefore protein detection would be disturbed.

4.3.6. Immunostaining
4.3.6.1. Paraffin embedding, tissue mounting and sectioning

Tissue samples were fixed in 4 % (w/v) PFA in PBS over night and washed thoroughly in PBS. Placed in plastic embedding cassettes (Tissue-Tek, Miles, USA) samples were dehydrated in an ascending ethanol series (Roth, Germany) from 50 % (v/v) to 96 % (v/v). According to their size, the samples were incubated for 2-6 hrs in each alcohol solution at 4 °C. Subsequently the samples were incubated twice for 2-6 hrs in isopropanol at 4 °C. The alcohol was exchanged by an isopropanol-paraffin mixture (1:1) for 24 hrs at 60 °C and the samples were transferred for at least 24 hrs to pure paraffin at 60 °C. The tissue was finally embedded in paraplast (Paraplast plus, Roth, Germany) using a paraffin embedding station (Leica, EG 1150H and Leica EG 1150 C, Leica, Germany) and mounted on section blocks for preparing 4 µm sections with a Leica microtome (Leica Jury RM2055, Leica, Germany).

4.3.6.2. Cryo embedding, tissue mounting and sectioning

Tissue samples were embedded in Tissue-Tek® (Sakura Finetek USA) and frozen in liquid nitrogen and cross-sectioned with a cryo-microtom (Mikrom® HM 500 OM, Germany) in 10 µm sections.

4.3.6.3. Immunohistochemistry

Paraffin sections were deparaffinized for 30 min at 50 °C in a humid chamber and sequentially subjected to 10 min incubation in Rotihistol (Roth, Germany), 5 min in 100 %, 96 % (v/v), 70 % (v/v), 50 % (v/v) ethanol (Roth, Germany) and finally 10 min in PBS. The procedure of fixation may lead to masking of sample epitopes; to render these epitopes accessible to the primary antibody, physical and chemical barriers are removed by a process called unmasking. For that purpose the samples were incubated with protease XXIV (Sigma, USA, 0.05 % (w/v) in PBS) and hyaluronidase (Sigma,

USA, 0.1 % (w/v) in acetate buffer (pH 6)) for 6 min, respectively 60 min at 37 °C in a humid chamber. Each enzyme was washed thoroughly from the slides with PBS before unspecific binding sites were blocked with 5 % (v/v) normal goat serum (Dako, Denmark), 1 % (w/v) BSA (Biomol, Germany) and protease inhibitor (Complete Mini, 1:5, Roche, Germany) in PBS for 60 min at 37 °C. Appropriate first antibodies were diluted in blocking buffer and incubated over night at 4 °C. After washing with PBS, the secondary antibody was applied diluted in PBS and incubated at 37 °C for 1 hr.

Tyramide signal amplification (TSA™) was applied in some cases to enhance weak immuno reactive signals. This enzyme-mediated detection method utilizes the catalytic activity of horseradish peroxidase (HRP) to generate higher density labelling. For signal intensification endogenous peroxidase was quenched for 60 min at room temperature in quenching buffer followed by regular blocking and incubation with the first antibody. A secondary biotinylated antibody was applied diluted in blocking solution for 60 min followed by the HRP conjugate (1:100 in blocking solution) for 45 min at rt. For tyramide labeling the Alexa Fluor 488 tyramide working solution was diluted according to the manufacturer's protocol in amplification buffer and incubated for 10 min. All reagents were purchased from Molecular Probes, USA. Nuclei were counter-stained with DAPI (Molecular Probes, USA) for 10 min and the slides were washed again with PBS before they were mounted with fluorescence mounting medium (Dako, Denmark). Cryosections were fixed in ice-cold acetone (Fischar, Germany) for 10 min at 4 °C. Blocking and further staining procedures were performed as described above.

4.3.6.4. Immunocytochemistry

For immuno staining, cells were fixed for 10 min either in ice-cold acetone (Fischar, Germany) or in 4 % (w/v) PFA (AppliChem, Germany) with 0.1 % (v/v) Tween 20 (Sigma, USA) in PBS. Fixing solution was removed by washing with PBS three times for 5 min each. Before blocking unspecific binding sites with a solution of 1 % (v/v) normal goat serum (Dako, Denmark) in PBS, auto-fluorescence was quenched using 100 mM NH_4Cl in PBS for 10 min. This step was performed for phalloidin/vinculin staining only. The blocking was followed by incubation with the first antibody diluted in blocking buffer either over night at 4 °C or 1 hr at 37 °C. The secondary antibody, diluted in blocking buffer was left on the slides for 1 hr at 37 °C. The nuclei were stained with DAPI for 10 min and stress fibers with phalloidin for 30 min at 37 °C (both

Molecular Probes, USA). Slides were mounted in fluorescence mounting medium (Dako, Denmark).

All histological incubation steps were performed in humid chambers. The stained structures were visualized with confocal laser scanning microscopy (C1 confocal microscope, C4 camera and software Nikon, Germany). In case of immunocytochemical staining of focal adhesion contacts and stress fibres, the number of focal adhesion contacts and length were determined in five independent fields of view in vinculin/F-actin fluorescence stainings of four patients per inflammation stage and depicted in FC per cell and µm per contact.

4.3.6.5. Immunocytochemistry detecting α1 integrin chain

Glass cover plates were coated with poly-L-lysine (5 µg/ml, Sigma, USA), collagen I (10 µg/ml, Sigma, USA), and recombinant collagen XVI (10 µg/ml and 30 µg/ml) at 4 °C over night as described in 4.5.6. After washing with PBS, the cover plates were blocked with 0.1 % BSA (Biomol, Germany) in PBS for 1 hr at rt. ISEMF were suspended in DMEM (Invitrogen, UK) culture medium with cell specific additives and w/o 0.1 mM $MnCl_2$ (Merck, Germany). The cells were seeded in a density of 15,000 cells/ml onto the substrate-coated glass plates. After incubation for 6 hrs at 37 °C under 5 % CO_2 cells were washed with PBS, supplemented with 2 mM $MgCl_2$ and 2 mM $CaCl_2$, w/o 0.1 mM $MnCl_2$ and fixed with 4 % PFA (AppliChem, Germany) in the same buffer for 7 min at rt. After triple washing with PBS cells were blocked and permeabilized in blocking buffer containing 0.5 % (v/v) Tween 20 (Sigma, USA), 2.5 % (v/v) methanol and 5 % (v/v) normal goat serum (Dako, Denmark) in PBS for 1 hr. ISEMF were stained with rabbit anti-vinculin antibody (V4139, Sigma, USA) and mouse anti-α1 integrin mAb (MAB1973Z, Chemicon, USA) diluted in blocking buffer 1:50 and 1:500, respectively, for 2 hrs. Primary antibodies were detected with AlexaFluor 488-conjugated anti-(rabbit IgG) antibody and AlexaFluor 568-conjugated anti-(mouse IgG) antibodies (both Molecular Probes, USA). Secondary antibodies were diluted 1:500 in blocking buffer and incubated for 2 hrs. Incubation with primary and secondary antibodies was followed by three washing steps with PBS. Nuclei were stained with 300 nM DAPI (Molecular Probes, USA) for 10 min. Finally, cells were washed in PBS and mounted in Fluoromount mounting medium (Dako, Denmark). Pictures were taken with a confocal laser scanning microscope (Nikon, C1 confocal microscope and Nikon C4 camera and software, Germany).

4.3.6.6. Flow cytometric analysis (FACS)

Primary ISEMF from CD patients were harvested with accutase (PAA, Germany) and the cell pellet was resuspended in blocking solution containing 1 % (w/v) BSA (Biomol, Germany) and 1 % (v/v) normal goat serum (Dako, Denmark) in PBS for 30 min at 4 °C. The cell suspension was incubated with antibodies and with the corresponding isotype control, which was diluted in blocking buffer for 1 hr at 4 °C. Cells were washed with PBS and resuspended with the appropriate secondary antibody (fluorescent dye conjugated) for 1 hr. After washing, cells were resuspended in 500 µl PBS for FACS (FACScanto, BD, USA). Data analysis was performed with WinMDI 2.8 software.

4.3.7. Protein A purification of polyclonal antibodies

Here, the ability of protein A, a 42 kDa polypeptide derived from the cell wall of *Staphylococcus aureus* to specifically bind to the Fc region of various immunoglobulins was utilized. The antibody purification was achieved by binding the antibody at neutral to high pH and by elution upon a shift to low pH using Vivapure Protein A Mini spin columns from Vivascience AG, Germany. The equilibrated columns were loaded with 100 µl of antiserum diluted with 300 µl of binding buffer (0.5 M sodium chloride, NaCl, 10 mM potassium phosphate, pH 7.4, both AppliChem, Germany) and centrifuged at 800 x g for 5 min. The column flow-through was reloaded and the process was repeated five times. Afterwards the columns were washed once with 400 µl binding buffer. The antibody was eluted using a 0.1 M glycine/HCl buffer (pH 2.8, AppliChem, Germany) and centrifuged at 800 x g for 5 min. Immediately after elution, the antibody solution was neutralized with neutralization buffer (1 M Tris (AppliChem, Germany), pH 9.0). The solution was concentrated to 100 µl using Vivaspin 2 columns with a 10 kDa molecular weight cut off and conserved with 0.02 % (v/v) sodium azide (Sigma, USA). Purified antibody was stored at 4 °C.

4.3.8. Protein extraction

Cell lysis was performed using RIPA buffer (Sigma, USA) containing protease inhibitor (1 tablet per 6 ml buffer, complete mini, Roche Applied Science, Germany). Confluent cell layers were trypsinized and washed with PBS. After centrifugation, the cell pellet was resuspended in RIPA buffer and incubated on ice for 30 min with interim

vortexing. Insoluble material was removed by centrifugation at 10,000 x g for 10 min. The protein content of the cell lysate was determined using bicinochoninic acid protein assay (BCA, Pierce, USA) and stored at -20 °C until further use.

4.3.9.   2D-gelelectrophoresis

The two-dimensional gelelectrophoresis separates proteins according to their isoelectric point (IEP) and their molecular weight. The first dimension constitutes the isoelectric focussing (IEF). Here, the sample is applied to a polyacrylamid strip (IPG), where a pH gradient is immobilized. After application of current, the proteins move along the gel and accumulate at their isoelectric point. At this point the overall charge of the protein is neutral. In the second dimension the proteins are separated according to their size by SDS-PAGE.

4.3.9.1.   Sample preparation, rehydration and sample loading

Aliquots of lysis buffer were thawed and protease inhibitor (complete mini, Roche, Germany) was added in a ratio of 1:20. The prepared lysis buffer was used once and additional freeze-thaw-cycles were avoided.

Cells were harvested using trypsin/EDTA (PAA, Germany) and washed with PBS. Per 0.8×10^6 cells 60 µl of lysis buffer was added. Suspended cells were vortexed and kept on a shaker for 15 min at 4 °C. Finally, lysed cells were centrifuged for 10 min at 10,000 x g at 4 °C and the clear supernatant was aliquoted and frozen at -80 °C for further usage after BCA protein quantification.

Rehydration and incubation of the sample, immobilized on an IPG strip were combined in one step. For that purpose, total protein with a concentration of 300 µg was diluted in rehydration buffer to a final volume of 125 µl. For a better orientation 2.5 µl of 2D-SDS-PAGE protein standard (BioRad, Germany) was added. Electrodes were prepared with pre-wetted electrode wicks and the sample mix was applied without air bubbles into an incubation chamber. The protective film was removed from the IPG strips and these strips were placed with the acrylamid side towards the sample. To avoid drying of the protein sample mineral oil covered the strips. The rehydration and focussing was performed in a Protean IEF Cell (BioRad, Germany) according to the following protocol. Buffer components and concentrations are listed in table 4-11.

4.3.9.2. Isoelectric focussing

Isoelectric focussing was performed according to a protocol (table 4-10) recommended by the manufacturer.

Step
Incubation of the protein; ramp voltage
Rehydration of a 7 cm IPG gel stripe at 10 °C
Active rehydration at 10 °C (12 hrs)
Pre-focussing at 250 V (15 min)
Focussing up to 4000 V (2.5 hrs)
Hold at 4000 V until 22,000 Vhrs are reached
Hold at 500 V to avoid diffusion

Table 4-10: Program of isoelectric focussing (IEF)

4.3.9.3. Equilibration of IPG strips for SDS-PAGE

After focussing the strips were washed thoroughly with water to remove mineral oil residues. The strips were subsequently incubated in equilibration solution containing 1 % (w/v) DTT (AppliChem, Germany) and 260 mM iodacetamid (BioRad, Germany). Both solutions were freshly prepared and used to avoid striation. The strips were placed bubble free into SDS gels together with 20 µl of loading dye and fixed with pre-warmed agarose (BioRad, Germany). The gels were run with 20 mA per gel for about 1 hr. The gels were finally stained with coomassie brilliant blue or with silver nitrate solution and scanned for further analysis with PD Quest software (BioRad, Germany).

Lysis buffer	9 M	Urea
	1 % (w/v)	DTT
	2 % (w/v)	CHAPS
	0.8 % (v/v)	Bio-lyte, pH 3-10 (BioRad, Germany)
	5 mM	Complete mini (Roche, Germany)
Rehydration buffer	8 M	Urea
	10 mM	DTT

	1 % (w/v)	CHAPS
	0.25 %	Bio-lyte, pH 3-10 (BioRad, Germany)
Equilibration buffer	6 M	Urea
	30 % (v/v)	Glycerol
	2 % (w/v)	SDS
		in 0.05 M Tris / HCl pH 8.8

Table 4-11: Buffer concentrations for lysis, rehydration and equilibration used in 2D-gelelectrophoresis

4.4. Scanning electron microscopy (SEM)

The scanning electron microscope images a sample surface by scanning it with a high-energy beam of electrons. The resolution is limited to 2 nm.

Cells were subjected to scanning electron microscopy to visualize their adhesion properties. For this purpose the cells (10,000 cells/cm²) were left to adhere in their culture medium for 3 hrs on pre-coated glass slides and were fixed for 20 min using 2.5 % (v/v) glutaraldehyde (Merck, Germany). The samples were subsequently dehydrated in ascending ethanol series (Roth, Germany) from 30 % (v/v) to 100 % (v/v) three times each for 20 min. Since air drying causes deformation of structure due to surface tension originated at the phase boundary when liquids evaporate, the samples were critical point dryed (CPD). The medium in this procedure is liquid CO_2 and the drying takes place above the critical point of this liquid medium at 73.8 bar and 31 °C. Dehydration medium and inter-medium in the critical-point-dryer (Balzers Union, Liechtenstein) was 100 % ethanol (Roth, Germany). The samples were kept in dry atmosphere in a desiccator after CPD until they were analyzed with scanning electron microscopy.

Samples were gold-sputted and visualized by a Hitachi S-2700 scanning electron microscope and evaluated using Digital Image Processing system 2.5 software.

4.5. Cell culture methods

4.5.1. Transfection

4.5.1.1. General transfection protocol

Transfection was carried out using Lipofectamine 2000 (Invitrogen, UK), a lipid-based transfection reagent. Cells were seeded at a density of 500,000 cells per well in a 6-well plate. Each reaction was incubated for 5 min with 5 µl Lipofectamine 2000 and 15 µl culture medium without FCS. cDNA (~ 0.5 µg/rct) was separately prepared with FCS-free medium up to a volume of 20 µl. Both solutions were gently mixed and incubated for 15 min at rt. Afterwards, the reaction volume was added to the cells together with usually applied culture medium and were incubated for 24 hrs at 37 °C and 5 % CO_2. After this time, medium was replaced by selection medium containing an appropriate antibiotic in a concentration previously determined in a kill curve for non-transfected cells. Transfection was controlled by transfecting the empty vector and by applying selection medium to non-transfected cells. By the time all non-transfected cells have died upon antibiotic application, resistent clones were isolated and further cultivated.

4.5.1.2. Transfection protocol for generation of shRNA expression retrovirus

Endotoxin-free retroviral vector, containing shRNAs, was transfected into the EcoPak 293-2 packaging cell line with Lipofectamine 2000 (Invitrogen, UK) as described above. After 24 hrs 6 µg/ml of puromycine (Biomol, Germany) was added. The antibiotic containing medium was renewed three times a week for a total of 10 days. Puromycin resistent clones were selected and transferred to a 25 cm² flask and further passaged to obtain enough transfected cells for transduction experiments. Transfected cells were also harvested for long time storage in liquid nitrogen and for transduction experiments (described in 4.5.4). For transduction, the culture medium containing virus was collected after 48 hrs from confluent flasks.

4.5.2. Titration of antibiotic stocks (kill curve)

Prior to application of puromycin in order to establish stable cell lines, selection agent stocks had to be titrated to determine the optimal concentration for selection with the chosen cell line. It is also important due to lot-to-lot variation of agent efficiency. 50,000 non-tranfected cells per well were plated in 6-well plates containing the culture medium plus increasing amounts of puromycin (0, 1.0, 2.5, 5.0, 7.5, and 10.0 µg/ml).

The cells were incubated for 10 days to 14 days and the selection medium was replaced every three days. For selecting stable transformants, the lowest concentration that induces massive cell death in about 5 days and kills all the cells within two weeks was used.

4.5.3. Virus titer determination

For virus titer determination 50,000 cells per well were seeded in a 6-well plate and cultured over night. The next day virus was added in a 10-fold serial dilution in complete medium containing 6 μg/ml polybrene. After 24 hrs of cultivation puromycin was applied in a previously determined concentration (see 4.5.2). Medium with antibiotic was renewed every second day. Control wells which contained non-transduced cells were treated with the same antibiotic concentration. After these cells had died the colonies of cells per well (colony forming units, cfu) for different virus concentrations were counted and the virus titer was determined according to this formula:

$$\text{cfu x dilution factor} = \text{cfu/ml}$$

4.5.4. Transduction of NIH3T3 cells

For transduction, culture medium containing shRNA expressing virus (without selecting antibiotic) was collected after 48 hrs of culture from confluent flasks. Virus titer was determined as described above and virus medium, characterized by at least 10^5 CFU was used for transduction. NIH3T3 cells ranging from 5×10^4 to 1×10^5 cells were seeded per well containing 1 ml of fresh or frozen (-80 °C) filtered (0.45 μm filter, Millipore, Germany) virus medium plus 6 μg/ml polybrene (Sigma, USA) and incubated over night at 37 °C and 5 % CO_2. Subsequently 1 ml fresh NIH3T3 growth medium (described in 4.5.5.2.1) containing puromycin in a final concentration of 5 μg/ml was added. The remaining colonies were transferred to larger flasks and further cultivated. Finally, cells were harvested by trypsin digestion and used for gene and protein analysis.

4.5.5. Cell source, preparation and culture conditions of the utilized cells

The table below lists all utilized cells, further described in this chapter.

Primary cells	Cell lines	
human	murine	human
ISEMF	NIH3T3	o CCD18Co
		o EcoPack2-293
		o HEK 293 EBNA
		o HEK 293 EBNA Strep16

Table 4-12: Cell types

4.5.5.1. Primary cells – human intestinal subepithelial myofibroblasts (ISEMF)

ISEMF were isolated from intestinal tissue, obtained from Crohn's Disease (CD) patients showing different inflammatory affection and histology. The degree of inflammation was graded endoscopically according to the following parameters shown in table 4-13. Determination of inflammation stage was also performed by hematoxylin and eosin staining and counting of neutrophils, eosinophils and lymphocytes.

Pat. No.	Sex	Degree of inflammation	Region of resection	Col XVI gene & protein	Adhesion	Migration	Proliferation	FACS scan	Focal adhesion count & length
201	♂	-	Colon ascendens	x	x	x	x	x	x
202	♀	-	Colon transversum		x		x	x	
206	♀	-	Colon ascendens	x	x		x	x	x
207	♂	-	Colon ascendens	x	x	x	x	x	x
210	♂	-	Colon ascendens	x	x	x	x	x	x
68	♂	-	Colon transversum						x
60	♂	+	Colon transversum						x
30	♂	+	Caecum	x	x		x	x	x
63	♂	+	Sigma	x	x	x	x	x	x
169	♀	+	terminal Ileum	x	x		x		
24	♂	+	Rectum	x	x	x	x	x	x
35	♂	+	Sigma		x	x		x	
62	♂	++	Colon ascendens	x	x	x	x	x	x
32	♂	++	Ileocaecum	x	x	x	x	x	x
171	♀	++	colon	x	x	x	x		
185	♂	++	terminal Ileum		x		x	x	

167	♀	++	Ileocaecum	x					x
192	♂	++	Sigma	x	x		x	x	x
69	♀	+++	Rectum	x	x			x	x
111	♀	+++	Sigma	x			x		
131	♀	+++	terminal Ileum		x		x		
190	♀	+++	Sigma	x	x	x	x	x	x
197	♂	+++	Caecum	x	x	x	x	x	
209	♂	+++	terminal Ileum	x	x	x	x	x	x

Table 4-13: Degree of inflammation, region of resection and relation of analyzed parameter of patient cell biopsies.

[-] non-inflamed (without pathological findings), [+] slightly inflamed (single small aphthous lesions), [++] inflamed (multiple aphthous lesions and small ulcers), [+++] highly inflamed (large ulcerous lesions)

Mucosal samples were completely denuded of epithelial cells by treatment with 2 mM EDTA (AppliChem, Germany) at 37 °C for 20 min and myofibroblasts were isolated according to established protocols and characterized by anti-vimentin and anti-vinculin staining (Mahida et al., 1997; Rogler et al., 2001). Cells were maintained in SM-GM (Table 4-17).

DMEM (Dulbecco's modified eagle's medium), Gibco, BRL, UK
10 % (v/v) FCS, Invitrogen, UK
1 % (v/v) penicillin-streptomycin, PAA, Germany
1 mM sodium pyruvate, Sigma, USA
1 % (v/v) non essential amino acids, Gibco BRL, UK
50 mg/ml gentamicin, Sigma, USA
8 µg/ml ciprofloxacin, Bayer, Germany
1 µg/ml amphotericin, Bristol-Meyers Squibb, USA

Table 4-14: Intestinal subepithelial myofibroblast growth medium (SM-GM).

4.5.5.2.   Cell lines
4.5.5.2.1. Murine cell lines

NIH3T3 fibroblasts (American Type Culture Collection (ATCC), no. CRL 1658) have been established from primary mouse embryonic tissue cultured by a designated protocol, so-called '3T3 protocol'. The spontaneously immortalized cells with a stable growth rate were established after 20-30 generations in vitro.

DMEM F-12, Gibco BRL, UK
10 % (v/v) FCS, Invitrogen, UK
1 % (v/v) penicillin-streptomycin, PAA, Germany

Table 4-15: NIH3T3 growth medium (NIH-GM).

4.5.5.2.2. Human cell lines
4.5.5.2.2.1. CCD18Co

These cells were originally derived from a colonic mucosal biopsy of a 2-month old female human infant and obtained from ATCC, no. CRL-1459.

EMEM (Eagle's minimal essential medium), Cambrex, Belgium
10 % (v/v) FCS, Invitrogen, UK
1 % (v/v) penicillin-streptomycin, PAA, Germany
1 mM sodium pyruvate, Sigma, USA
0.1 mM non essential amino acids, Gibco BRL, UK

Table 4-16: CCD18Co growth medium (CCD18Co-GM).

4.5.5.2.2.2. EcoPack2-293

The adherent BD EcoPack2-293 packaging cell line (BD, Bioscience, USA) were derived from the human embryonic kidney (HEK 293) cell line and was designed for rapid, transient and also stable production of ecotropic retrovirus. Virus produced by these cells possesses an ecotropic envelope (gap70) and was therefore only able to infect mouse and rat cells.

DMEM F-12, Gibco BRL, UK
10 % (v/v) FCS, Invitrogen, UK
1 % (v/v) penicillin-streptomycin, PAA, Germany
4 mM L-glutamine, PAA, Germany
1 mM sodium pyruvate, Sigma, Germany

Table 4-17: EcoPack2-293 growth medium (ECO-GM).

4.5.5.2.2.3. HEK 293 EBNA

The HEK 293 EBNA cell line is a human embryonal kidney cell line derived from HEK 293 (ATCC, no. CRL-1573). This cell line was stably transfected with the Epstein Barr virus (EBV) nuclear antigen 1 (*EBNA-1*) gene. These cells propagate plasmids containing an EBV replication origin and keep it episomal (Young et al., 1988). The expression of the *EBNA-1* gene is controlled by a CMV promoter and combined with a neomycin resistence gene (Lambert et al., 1988). Therefore, the cell line has to be cultured permanently with geneticin (G418), a neomycin analogon.

DMEM F-12, Gibco BRL, UK
10 % (v/v) FCS, Invitrogen, UK
1 % (v/v) penicillin-streptomycin, PAA, Germany
175 µg/ml geneticin (G418), PAA, Germany

Table 4-18: HEK 293 EBNA growth medium (EBNA-GM)

4.5.5.2.2.4. HEK 293 EBNA Strep 16 cells

These cells were derived from the HEK 293 EBNA cell line transfected with the expression vector pCEP Pu BM40SP C-StrepII to stably express recombinant collagen XVI.

Growth medium for transfected cells was EBNA-GM supplemented with 0.5 µg/ml puromycin (Biomol, Germany).

4.5.6. *Proliferation assay*

96-well plates (Falcon, USA) were coated with poly-L-lysine (5 µg/ml, Sigma, USA), collagen I (10 µg/ml, Sigma, USA) and recombinant collagen XVI (Kassner et al., 2004) (10 µg/ml and 30 µg/ml) over night at 4 °C. Unspecific binding sites were blocked with 1 % (w/v) BSA (Biomol, Germany) solution for 2 hrs at rt. The plates were washed two times with PBS and stored at 4 °C until usage. Cells were trypsinized and cultivated over night at 37 °C and 5 % CO_2 at a density of 7,500 cells/well. EZ4U (Biomedica, Austria) proliferation assay was performed according to the manufacturer's protocol for 2 hrs. In this assay tetrazolium is reduced to formazan by active succinat

dehdrogenase of living cells. This reduction process requires functional mitochondria which are inactivated within a few minutes after cell death. Absorption was determined at 450 nm and a reference wavelength of 600 nm.

4.5.7. Adhesion assay

96-well plates were coated as described above and ISEMF were seeded at a density of 7,500 cells/well into the plates. Cells were allowed to adhere for 30 min and 60 min at 37 °C at 5 % CO_2. Subsequently, plates were washed with PBS in order to remove non-adhered cells. Adhered cells were fixed with 1 % (v/v) glutaraldehyde solution (Merck, Germany) for 30 min. Glutaraldehyde was removed and cells were washed with PBS again. Fixed cells were stained with 0.02 % (w/v) crystal-violet solution (Sigma, Germany) for 15 min at rt and washed with tap water. Bound crystal violet was removed by incubation for 3 hrs at a horizontal shaker at 100 rpm with 70 % (v/v) ethanol (Roth, Germany). Crystal violet adsorption was measured at 600 nm and each sample was performed in triplicates.

4.5.8. Migration assay

ISEMF were seeded at a density of 20,000 cells/well into a 24-well plate and grown to confluency at 37 °C and 5 % CO_2. Confluent cell layers were scratched with a 100 µl pipet tip and cells were supplied with fresh cell culture medium. The width of the scratch was determined after 0 hrs, 2 hrs, 6 hrs and 24 hrs and related to the covered surface. Completely covered cell culture surface referred to 100 % whereas the wound at 0 hrs referred to 0 %.

4.5.9. Live/dead assay

Living cells can be distinguished from dead cells by the presence of intracellular esterase activity which was determined by the enzymatic conversion of cell-permanent calcein acetoxymethylester (calcein AM) to the fluorescent calcein. This dye is retained in living cells and can be detected by fluorescent emission (ex/em ~495 nm/~515 nm). Ethidium homodimer (EthD-1) enters only cells with a defect membrane. Fluorescence is enhanced upon binding to nucleic acids, producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). This dye is excluded by the intact plasma membrane of living cells.

Cells were cultured subconfluently in 96-well plates and washed with PBS to remove medium residues. 0.025 % (v/v) calcein and 0.05 % (v/v) EthD-1 (both Molecular Probes, Germany) were solubilized in PBS. Per well, 300 µl of this mixture were added and incubated in the dark at 37 °C and 5 % CO_2. After 45 min the solution was removed and the cells were washed with PBS. The substrate toxicity was determined by the ratio of dead to living cells.

4.6. Preparation of recombinant collagen XVI in HEK 293 EBNA cells

4.6.1. Episomal expression system of collagen XVI in HEK 293 EBNA cells in roller flask culture

To obtain high amounts of recombinant collagen XVI plastic roller flasks with an inner surface of 2125 cm² were used to culture HEK 293 EBNA Strep 16 cells. The cells were originally passaged from 75 cm² flasks in a ratio of 1:5 into 175 cm² flasks. After reaching confluence, cells obtained from two flasks were transferred to one roller flask containing 100 ml of culture medium. Defined gas exchange was guaranteed under sterile conditions with 5 % CO_2 and roller flasks were tightly closed and incubated at 37 °C in a temperature controlled rolling incubator (0.5 - 0.7 rpm). Serumfree medium for generation of conditioned medium was supplemented with 175 µg/ml geniticin (PAA, Germany).

DMEM F-12 (Dulbecco's modified eagle's medium), Gibco BRL, UK
256 µM ascorbic acid, Sigma, USA
1 mM ascorbate-2-phosphate, Sigma, USA
1 mM sodium pyruvate, Sigma, USA
1 mM cystein, Sigma, USA
1 % (v/v) penicillin-streptomycin, PAA, Germany

Table 4-19: Serum-free medium (SFM)

4.6.2. Generation of recombinant collagen XVI containing conditioned medium

HEK 293 EBNA Strep 16 cells were cultured in serum containing medium for 48 - 54 hrs and finally the medium was replaced by 50 ml serum-free medium (SFM) per flask. The conditioned medium was collected and centrifuged (10 min at room temperature, 3540 x g) after 48 hrs and replaced with fresh medium. The supernatant was mixed with

avidin (Merck, Germany) in a final concentration of 833 µg/l and kept at 4 °C until it was applied to affinity chromatography for protein purification. The generation of conditioned medium was repeated up to six times with the same cells.

4.6.3. Purification of recombinant collagen XVI

Recombinantly produced collagen XVI was purified employing an oligo peptide, the C-terminal StrepII-Tag (comprising of the sequence WSHPQFEK) with a high affinity to Strep-Tactin that way allowing affinity purification of the protein.

4.6.4. Affinity chromatography with Strep-Tactin columns

Fusion proteins containing a StrepII-Tag bind tightly to a modified streptavidin called Strep-Tactin (Skerra and Schmidt, 2000) immobilized on an affinity column. The elution of the bound protein is enabled by competition to desthiobiotin that binds reversibly to the column matrix. Columns are regenerated by exchanging desthiobiotin with the azo dye HABA (2-(4-hydroxyphenylazo)benzoe-acid). Recombinant collagen XVI was purified according to the manufacturer's protocol (IBA, Germany). The elution of the protein was performed with pre-chilled buffers and the following dialysis against sterile PBS was performed at 4 °C.

The collagen XVI conditioned medium was mixed with avidin (833 µg/ml) to form an avidin-biotin complex and cooled down to 4 °C. Before medium was loaded on to the affinity column, the initial volume was mixed with 0.1 volumes of buffer W (10x). The prepared medium passed the pre-equilibrated (100 ml buffer W) column, just with gravity force. Afterwards, the column was washed with 50 ml buffer W prior to elution with 25 ml buffer E which was monitored by an UV detector (Uvicord SII, Amersham Biosciences) with a one channel recorder (R111, Amersham Biosciences). The eluted protein was fractionated into 1.5 to 2 ml aliquots. Fractions were pooled according to the protein content and dialysed against sterile PBS. Protein concentration was determined using the BCA assay and approved by immunoblotting.

Regeneration of the column material was performed with 50 ml buffer W, followed by 100 ml buffer R. The matrix was prepared for repeated chromatography by washing with 100 ml buffer W. All buffer components and concentrations are listed in table 4-20.

Buffer	Components
Buffer W	100 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0, all AppliChem, Germany
Buffer W (10x)	1 M Tris, 1.5 M NaCl, 10 mM EDTA, pH 8.0, all AppliChem Germany
Buffer E	Buffer W + 2.5 mM desthiobiotin, Sigma, USA
Buffer R	Buffer W + 1 mM HABA (Fluka, Germany)

Table 4-20: Buffer concentrations for affinity chromatography utilizing Strep-Tactin columns

4.7. Statistical analysis

Results are shown in average values ± standard error of mean. In experiments with ISMEF, for each inflammation stage at least 4 patients were analyzed in triplicates. A detailed patient list and related experiments is found in table 4-13. In gene silencing experiments significances are calculated for three independent knockdown cell batches. Significances were determined with assistance of GraphPadPrism v.5 by one-way Anova analysis of variance with Tukey's multiple comparison test for the proliferation and student's t-test for adhesion assays and integrin expression profile. In gene silencing and downstream experiments one-way Anova analysis of variance with Tukey's multiple comparison test was applied.

5. Results

What we think, or what we know, or what we believe is, in the end, of little consequence. The only consequence is what we do. – John Ruskin

Part I: The role of collagen XVI in pathological disorders

5.1. Recombinant expression of collagen XVI in HEK 293 EBNA cells

The expression system of collagen XVI in HEK 293 EBNA cells has been established previously (Kassner et al., 2004). However, due to passaging, the transfected cells had gradually lost their potential to efficiently express collagen XVI. Therefore, new cell batches were transfected with pCEP Pu vector encoding for the alpha1 chain of collagen XVI.

5.1.1. Purification of recombinant collagen XVI

Transfected cells secrete collagen XVI which can be purified with affinity chromatography based on a Strep-Tactin–StrepII-Tag binding to the C-terminal StrepII-Tag. Immunoblotting with an antibody against collagen XVI in figure 5-1 shows protein fragments of 213 kDa, 182 kDa, 133 kDa and 78 kDa whereas immunoblotting with an antibody against the StrepII-Tag does not display the 133 kDa fragment.
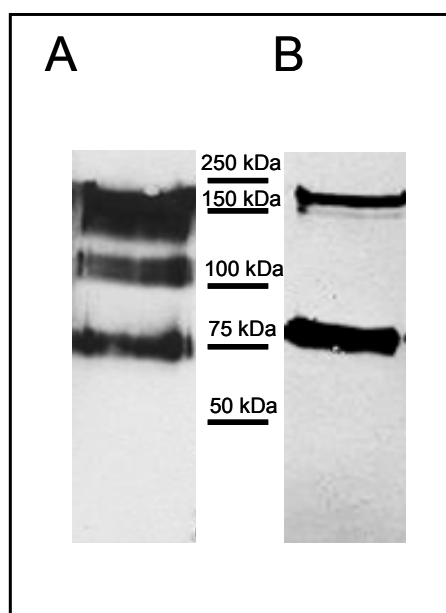

Figure 5-1: Immunoblots of serum-free conditioned medium from transfected HEK 293 EBNA cells using antibodies against collagen XVI (A) and StrepII-Tag (B).
100 µl serum-free medium, conditioned for 48 hrs on a 75 cm² cell culture flask was applied.

Recombinantly produced collagen XVI was isolated from at least 400 ml conditioned medium of roller cultures. Affinity purification was performed on a 10 ml Strep-Tactin Superflow column. 5 to 10 ml of protein solution with 90 to 150 µg/ml protein was obtained per purification cycle which refers to an average total protein amount of 3.0 mg collagen XVI per liter conditioned medium.
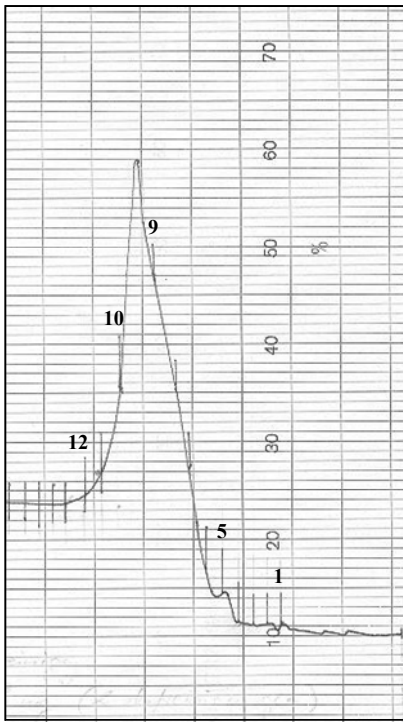

Figure 5-2: Standard elution profile obtained from affinity purification of recombinant collagen XVI using a Strep-Tactin column of 10 ml volume. Elution was performed with 50 ml elution buffer containing 2.5 mM desthiobiotin. 1.5 ml fractions were collected. The peak fractions (#6 to #12) were pooled and dialyzed. One elution resulted in 600 µg to 1200 µg recombinant collagen XVI. The elution profile was recorded with a detector sensitivity of 0.1 V.

5.1.2. Long-term storage of recombinant collagen XVI

The storage of recombinant collagen XVI was performed as a 50 % glycerol stock solution at -80 °C after shock freezing in liquid nitrogen or as lyophilized protein at -80 °C. Collagen XVI stored at 4 °C adheres to vessel surfaces and gradually degrades. Freezing at -20 °C destroys the collagen helix by crystallized ice layers formed during concentration dependent gradient freezing at this temperature.

5.2. Molecular distribution of collagen XVI in the human intestinal wall

Collagen XVI is a component of the extracellular matrix produced by keratinocytes and fibroblasts in skin (Grassel et al., 1999). Here, we focused on immunofluorescence staining of bowel tissue derived from Crohn's disease patients. We compared histological normal appearing tissue with diseased tissue represented by strictured areas. Immunofluorescence staining of intestinal sections with anti-α-smooth muscle actin and anti-collagen XVI antibodies revealed collagen XVI distribution throughout all bowel

layers. Collagen XVI is localized in the epithelial cell layer and matrix of the luminal surface and is produced by cells throughout the intestinal mucosa. In addition, mesenchymal cells of the lamina propria (Fig. 5-3 A, B, white arrow heads) and underlying muscular layers, here predominantly around blood vessels, stain positive for collagen XVI (Fig. 5-3 C, and D, white arrow heads). Collagen XVI is equally distributed on sections from CD patients within areas where the normal composition of bowel layers is still retained (Fig. 5-3 E, H). Strictures, a characteristic of the disease, lack distinct compartments and are comprised of the different intestinal tissues in varying composition. In these strictured parts (Fig. 5-3 F, G) collagen XVI positive cells appear more densely packed in the submucosa compared to healthy sections (Fig. 5-3 C). Collagen XVI seems closely restricted to single cells, however in strictures collagen XVI is deposited into the ECM and is found around cells (Fig. 5-3 E, F).
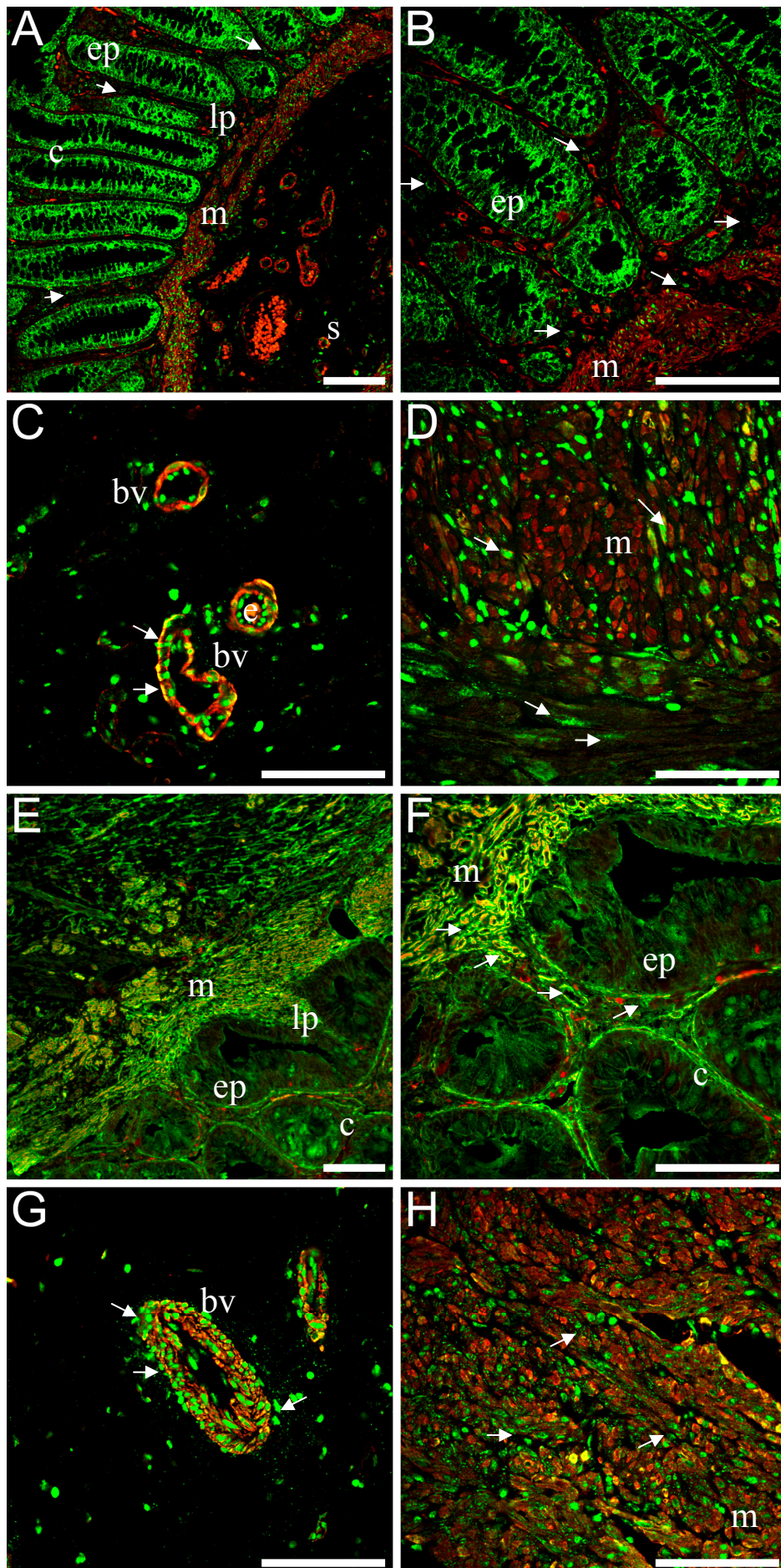
Figure 5-3: Morphological distribution of collagen XVI in healthy and CD tissue sections.

Morphological distribution of collagen XVI (green fluorescence) and α-smooth muscle actin (red fluorescence) in paraffin sections is depicted for the bowel wall of healthy tissue (A-D) versus CD tissue (E-H). Arrow heads indicate representative positive staining for collagen XVI. Bar represents 100 μm.

c: crypts, m: muscle layer, s: submucosa, bv: blood vessel, e: erythrocytes, ep: epithelial cells, lp: lamina propria

5.3. Distribution of α1- and α2 integrin in the human colon

Immunofluorescence staining of α1 integrin in the human colon revealed a co-localization with collagen XVI in the lamina propria, whereas α2 integrin is mainly expressed by epithelial cells of the crypts.

Figure 5-4: Morphological distribution of Collagen XVI and α1 integrin in colon tissue sections (cryo sections). Bar represents 100 μm.

Morphological distribution of collagen XVI (red fluorescence, A) and α1 integrin (green fluorescence, B) is depicted for the bowel wall. Collagen XVI and α1 integrin are colocalized (merge, C).
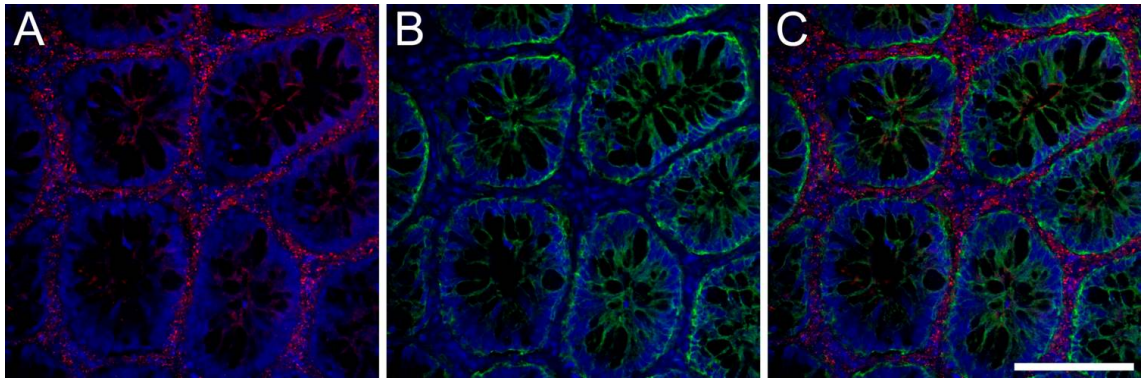
Figure 5-5: Morphological distribution of Collagen XVI and the integrin α2 in colon tissue sections (cryo sections). Bar represents 100 µm.
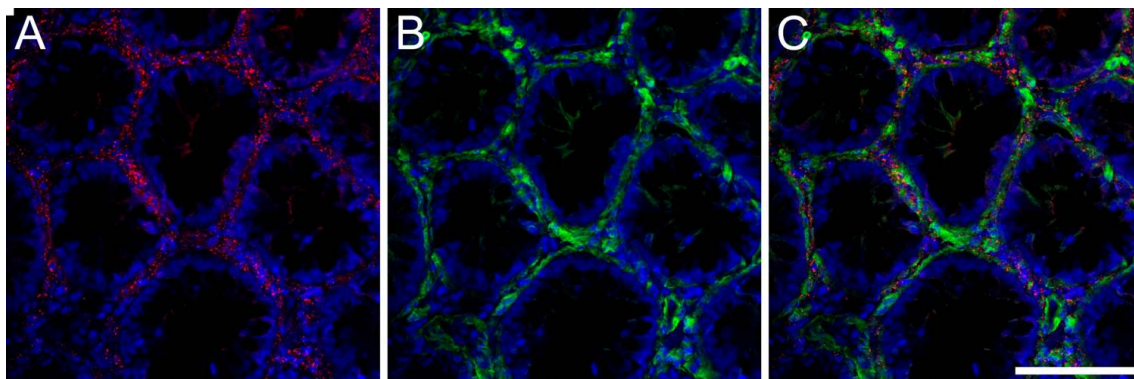
Morphological distribution of collagen XVI (red fluorescence, A) and α2 integrin (green fluorescence, B) is depicted for the bowel wall. Merge is depicted in C.

5.4. Intestinal subepithelial myofibroblasts (ISEMF) express collagen XVI

5.4.1. Gene expression analysis of collagen XVI in ISEMF and intestinal epithelial cells

ISEMF from each inflammation stage and intestinal epithelial cells showed collagen XVI gene expression in qualitative PCR (data not shown) as already demonstrated in immunohistochemistry in 5.2. Collagen XVI could also be detected in intestinal epithelial cells from healthy patient material.

5.4.2. Immunocytochemical analysis of ISEMF

ISEMF of all inflammation stages synthesized collagen XVI protein that was located all over the cellular body (Fig. 5-6). A matrix deposition of collagen XVI could not be detected during the cultivation period of 24 hrs. Co-staining with fibronectin revealed the same distribution in the cell with a higher concentration around the nuclei and no matrix deposition. ISEMF exhibit similar morphology irrespective of inflammation degree.
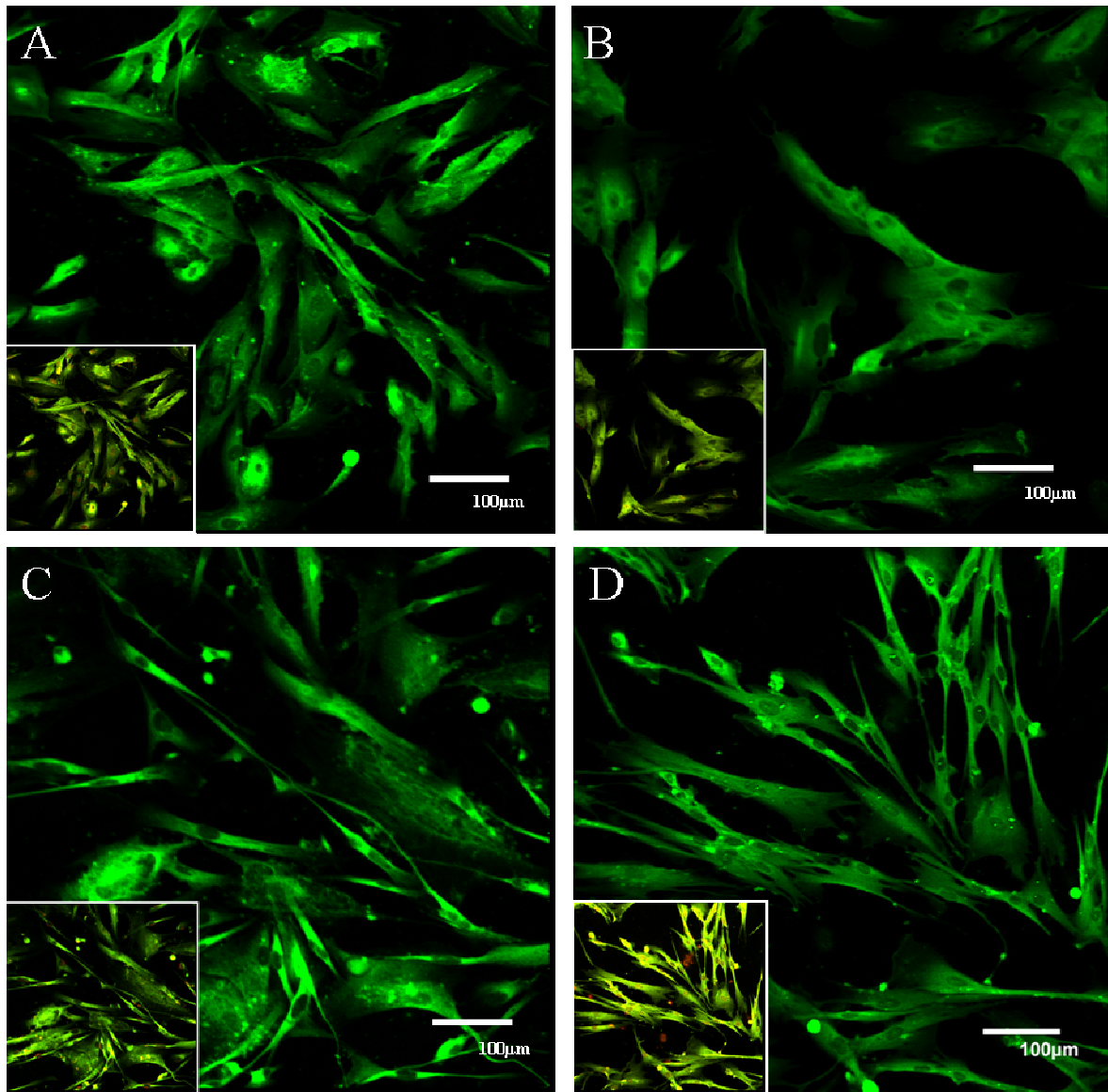
Figure 5-6: Immunocytochemical staining for collagen XVI (green).

Large pictures depict only collagen XVI protein, whereas inlets show merge of collagen XVI and fibronectin (yellow). All inflammation stages are shown in one representative image: non-inflamed (A), slightly inflamed (B), moderately inflamed (C) and highly inflamed (D).

5.4.3. Immunoblotting of ISEMF cell lysates (Western blotting)

Collagen XVI protein production was analyzed with immunoblotting of cell lysates. Increased levels of collagen XVI were found in moderately and highly inflamed cells compared to lower inflammation stages (Fig. 5-7 C). The CCD18Co cells synthesized clearly more collagen XVI as ISEMF from non- or slightly inflamed tissue biopsies. We detected proteolytically cleaved fragments of collagen XVI as reported for smooth

muscle cells and dermal fibroblasts (Grassel et al., 1996). Most prominently present was the ~ 180 kDa fragment besides the ~ 220 kDa, ~ 130 kDa and ~ 78 kDa fragments.
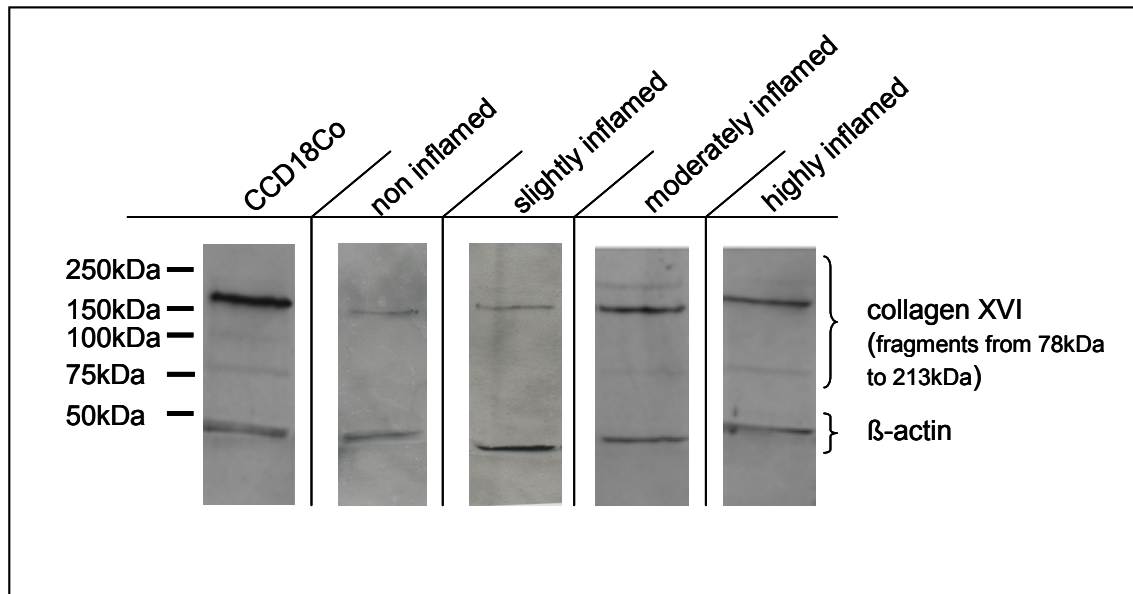
Figure 5-7: Immunoblotting of cell lysates for collagen XVI and ß-actin.
Immunoblotting of collagen XVI was performed with 20 µg RIPA protein lysates of ISEMF per lane after 24 hours culture in serum-free medium. For each inflammation stage one representative image is depicted. Bands between 75 kDa and 250 kDa represent collagen XVI (full length protein ~ 220 kDa); bands below 50 kDa represent ß-actin employed as loading control. For each inflammation stage cells from at least four patients have been investigated

5.4.4. *Integrin expression profile of intestinal subepithelial myofibroblasts*

Integrin expression profile of ISEMF was determined for the α1- and α2 integrin subunits (Fig. 5-8). Expression profiles of β1, α10 and α11 intergrin subunits have been investigated additionally, but revealed no significant changes on the myofibroblasts with respect to inflammation stage (data not shown). α1 integrin surface expression was very weak on non-inflamed (Fig. 5-8 B) and slightly-inflamed ISEMF (Fig. 5-8 C). However, increasing inflammation severity was significantly associated with increasing α1 integrin expression on the cell surface of ISEMF (Fig. 5-8 D, E) while α2 integrin expression remained unchanged in all stages of inflammation. Compared to the isotype control the CCD18Co cell line showed a clear increase of α1 integrin surface expression compared to α2 integrin (Fig. 5-8 A).
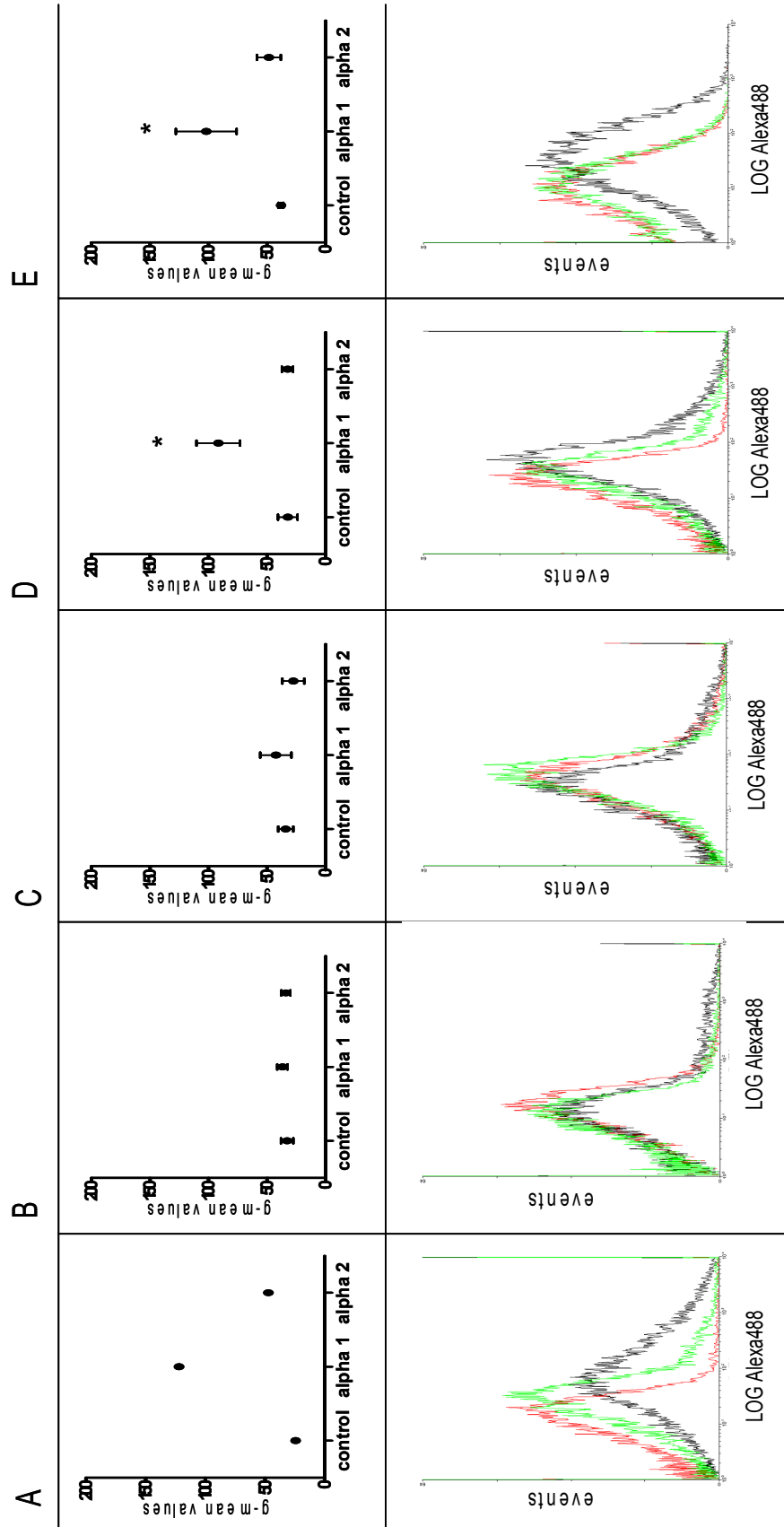
Figure 5-8: Cell surface expression of α1- and α2 integrin subunits on ISEMF and CCD18Co cells was determined using flow cytometry. Geometric mean (g-mean) of the fluorescence intensity was determined using WinMDI 2.8 software and shows integrin expression rates for the CCD18Co cell line (A), non-inflamed ISEMF (B), slightly inflamed ISEMF (C), moderately inflamed ISEMF (D) and highly inflamed ISEMF (E) always compared to the negative isotype control. The panel below shows a representative FACS histogram of one patient. n = 5 (non-inflamed), n = 4 (slightly inflamed), n = 4 (moderately inflamed), n = 4 (highly inflamed)
* $p<0.05$

5.4.5. *Effect of recombinant collagen XVI on the metabolism of intestinal subepithelial myofibroblasts*

5.4.5.1. Effect of recombinant collagen XVI on cell viability

Collagen XVI was applied in concentrations of 10 µg/ml and 30 µg/ml to tissue culture surfaces. Collagen XVI effect on cell viability was determined after over night culture of cells in a live/dead assay. Membrane-permeant calcein was cleaved by esterases in living cells which resulted in green fluorescence. The membrane-impermeant ethidium homodimer labeled nucleic acids of membrane-compromised cells and showed red fluorescence. With this assay no cytotoxic effect of collagen XVI in ISEMF could be detected.
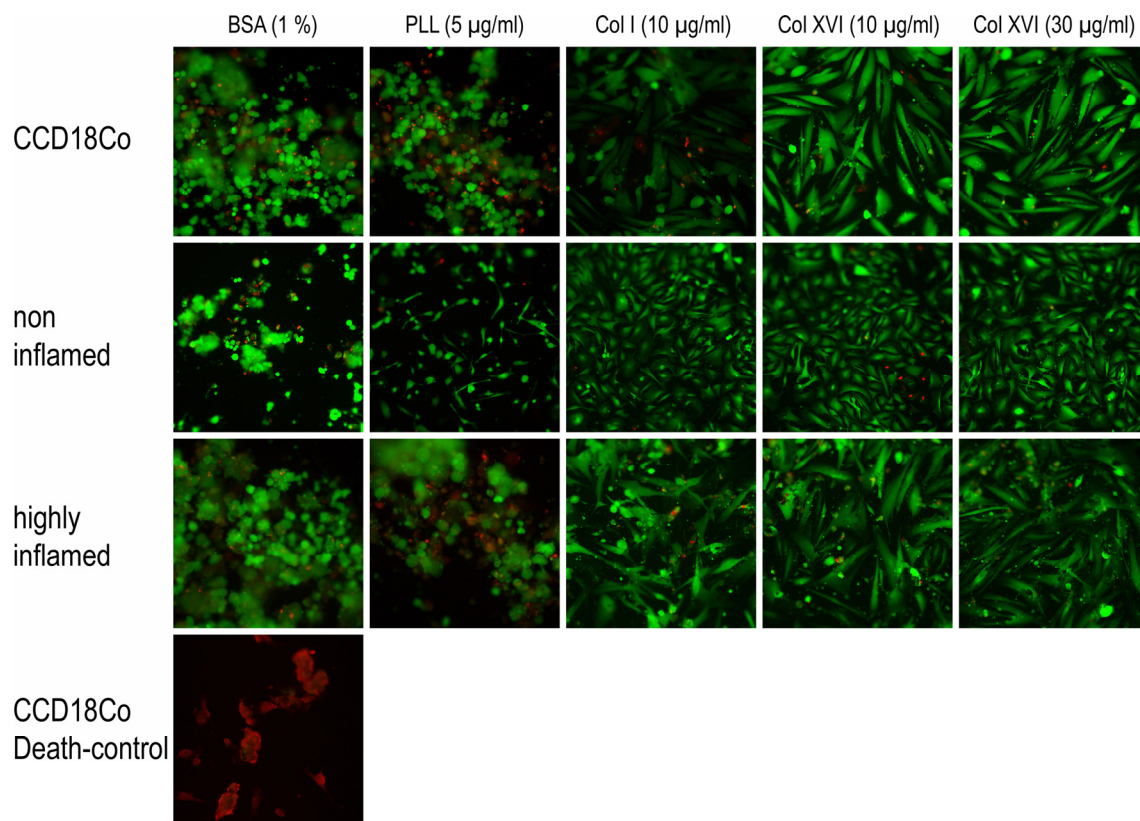
Figure 5-9: Live/dead assay on ISEMF. CCD18Co cells, non-inflamed and highly inflamed cells were stained with calcein (green fluorescence) for living cells and ethidium homodimer-1 (red fluorescence) for dead cells.

5.4.5.2.   Effect of collagen XVI on adhesion properties of ISEMF

Overall, ISEMF revealed a reduced adhesion capacity if derived from non-, slightly- and moderately inflamed tissue biopsies on all coatings compared to the CCD18Co cell line (Fig. 5-10 A-F). The CCD18Co cell line and non-inflamed ISEMF showed profoundly reduced adhesion capacities on collagen XVI compared to poly-L-lysine (PLL) and collagen I (Fig. 5-10 A, B). A comparable adhesion spectrum could be observed for moderately inflamed ISEMF, however without reaching significance (Fig. 5-10 D). Slightly inflamed cells adhere the weakest with no difference regarding the substrates. With increasing inflammation adhesion capacity on collagen XVI was unchanged with respect to PLL, collagens I and XVI (Fig. 5-10 C, D, E) however, there is a tendency towards less adhesion on collagen XVI (Fig. 5-10 D, E). Figure 5-10 A-D displays cellular adhesion spectra to different substrates and figure 5-10 F compares cell adherance tendency of different inflammtion stages to collagen I and collagen XVI

substrates. ISEMF obtained from moderately and highly inflamed tissue adhered stronger on collagen XVI as non-inflamed or slightly inflamed cells did (Fig. 5-10 F).
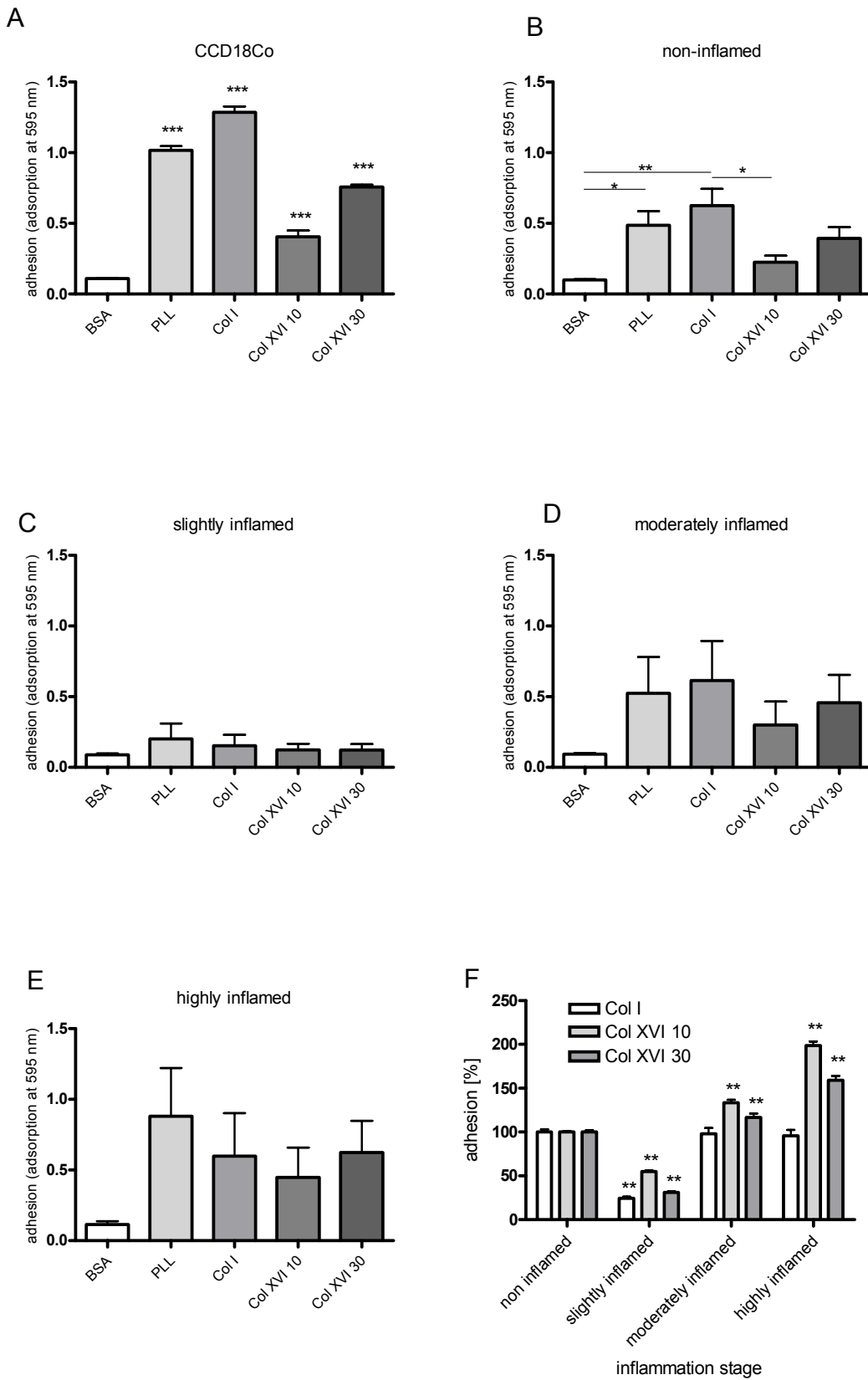
Figure 5-10: Adhesion properties of ISEMF

Adhesion was determined by crystal violet staining of CCD18Co (A) and ISEMF isolated from increasingly inflamed CD tissue biopsies (B-E) left to adhere on coated 96 well plates for 30 min. Adhesion was determined for differently inflamed ISEMF on following coatings: 10 mg/ml BSA; 5 µg/ml poly-L-lysine (PLL); 10 µg/ml collagen I (Col I); 10 µg/ml collagen XVI (Col XVI 10); 30 µg/ml collagen XVI (Col XVI 30). A-E display adhesion spectra of cells from one inflammation stage on different substrates and F displays adhesion tendency of cells from different inflammation stages on collagen I and collagen XVI. n = 5 for each inflammation stage
* p<0.05; ** p<0.01; *** p<0.001

5.4.5.3.   Effect of collagen XVI on cell spreading of ISEMF

A more pronounced cell spreading on collagen XVI compared to collagen I could be observed for ISEMF from all inflammation stages with scanning electron microscopy (Fig. 5-11).
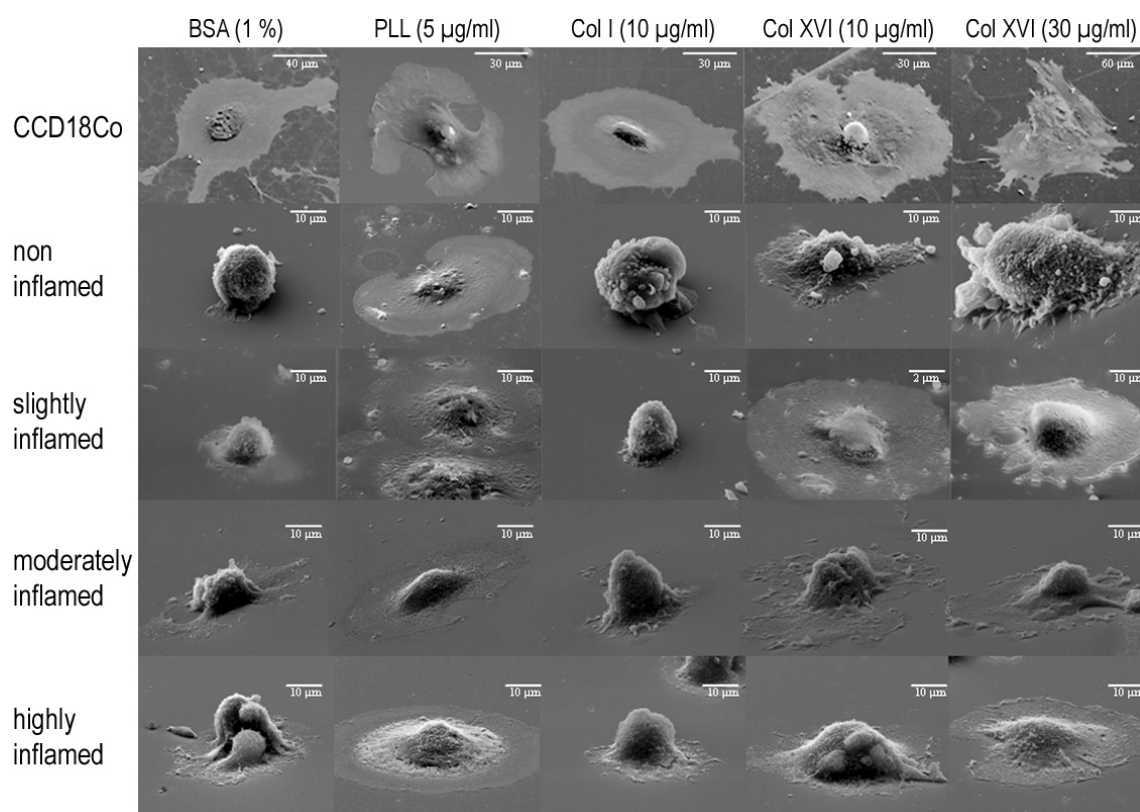
Figure 5-11: Cell spreading of ISEMF

ISEMF and CCD18Co cells were cultured with a density of 10,000 cells/ml on coated glass slides for 3 hrs and cell spreading and cell shape were optically evaluated by scanning electron microscopy. Cell spreading was promoted on poly-L-lysine and

collagen XVI compared to collagen I and the BSA. Representative images were depicted (n = 3).

5.4.5.4.   Effect of collagen XVI on cell number of ISEMF

Figure 5-12 displays data obtained by assaying proliferation of CCD18Co cells (Fig. 5-12 A) and ISEMF (Fig. 5-12 B) in all inflammation stages. Proliferation assay was performed analyzing total cell number seeded on 1 % (w/v) BSA, poly-L-lysine (5 µg/ml), collagen I (10 µg/ml), and two different concentrations of collagen XVI (10 µg/ml and 30 µg/ml). As ISEMF obtained from different inflammation stages did not display changes in their total cell number on all tested culture substrates, data for all inflammation stages were combined (Fig. 12 B). Cell line CCD18Co showed increased proliferation on both collagen XVI coating concentrations compared to collagen I and poly-L-lysine and thus presumably an increased proliferative rate.
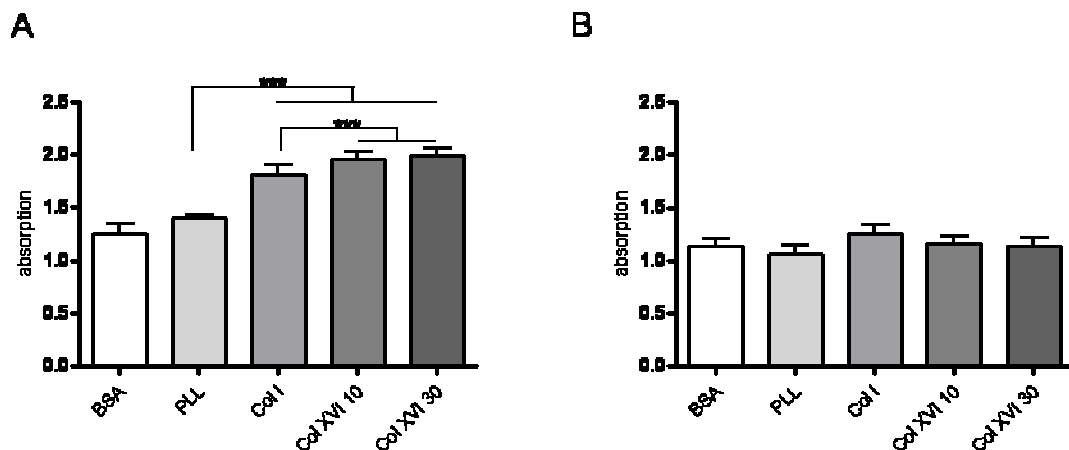
Figure 5-12: Total cell number of ISEMF.

Proliferation assay (EZ4U) was performed with ISEMF in different inflammation stages grown overnight on coated well plates. The cell line CCD18Co exhibited the highest total cell number on collagen XVI as substrate (A), while proliferation capacity of ISEMF from different inflammation stages remained unchanged. Data from these experiments were combined (B).

n = 5 (A); n = 14 (B); *** p<0.001

5.4.5.5.   Effect of collagen XVI on the migratory behaviour of ISEMF

The migration assay revealed no differences in migration properties according to inflammation stages nor according to culture substrates (data not shown).

5.4.5.6. Effect on the formation of focal adhesion contacts –
determination of quantity and maturation stage

Collagen XVI and collagen I promoted formation of vinculin containing focal adhesion contacts on ISEMF from all inflammation stages compared to poly-L-lysine and the BSA control (Fig. 5-13, 5-14) with the strongest focal adhesion contact formation on recombinant collagen XVI as substrate (Fig. 5-13 A-E). In general, higher counts of focal adhesion contacts refer to better cell spreading as demonstrated in figure 5-11. Overall, non-inflamed cells and cells from the early inflammatory phase showed elevated numbers of total focal adhesion contacts compared to moderately or highly inflamed ISEMF (Fig.5-13 A-E), as summarized for collagens I and XVI as substrates (Fig. 5-13 F). Figure 5-13 A-D displays focal adhesion formation spectra on different substrates and figure 5-13 F compares focal adhesion formation tendency on collagen I and collagen XVI substrates.

In addition, the length of vinculin containing focal adhesion contacts revealed a similar tendency as their amount (Fig. 5-15 A-F). Collagen I and collagen XVI induce maturation and, therefore length of focal adhesion contacts in all inflammation stages compared to poly-L-lysine. In ISEMF derived from all inflammation stages collagen XVI even appeared to promote formation of much significantly longer focal adhesion contacts compared to collagen I (Fig. 5-15 C-E). However, total length of focal adhesions decreased in inflamed ISEMF compared to non-inflamed on collagens I and XVI (Fig. 5-15 F). Figure 5-15 A-D displays focal adhesions length spectra on different substrates and figure 5-15 F compares focal adhesions lenght tendency on collagen I and collagen XVI substrates.

Figure 5-13: Formation of focal adhesion contacts – quantitation

CCD18Co cells and ISEMF were cultured over night on coated glass slides and stained with ALEXA fluorophores for actin stress fibers (phalloidin coupled antibody) and the presence of focal adhesion contacts (anti–vinculin antibody). Focal adhesion contacts were counted per cell sample and per inflammation stage in five independent fields of

view and referred to as focal adhesion contacts per cell. A-E display spectra of the amount of focal adhesions on different substrates and F displays the tendency of amount of focal adhesion contacts on collagens I and XVI during increasing inflammation. n = 5 (non-inflamed), n = 4 (slightly inflamed), n = 4 (moderately inflamed), n = 3 (highly inflamed)

*p<0.05; ** p<0.01; *** p<0.001
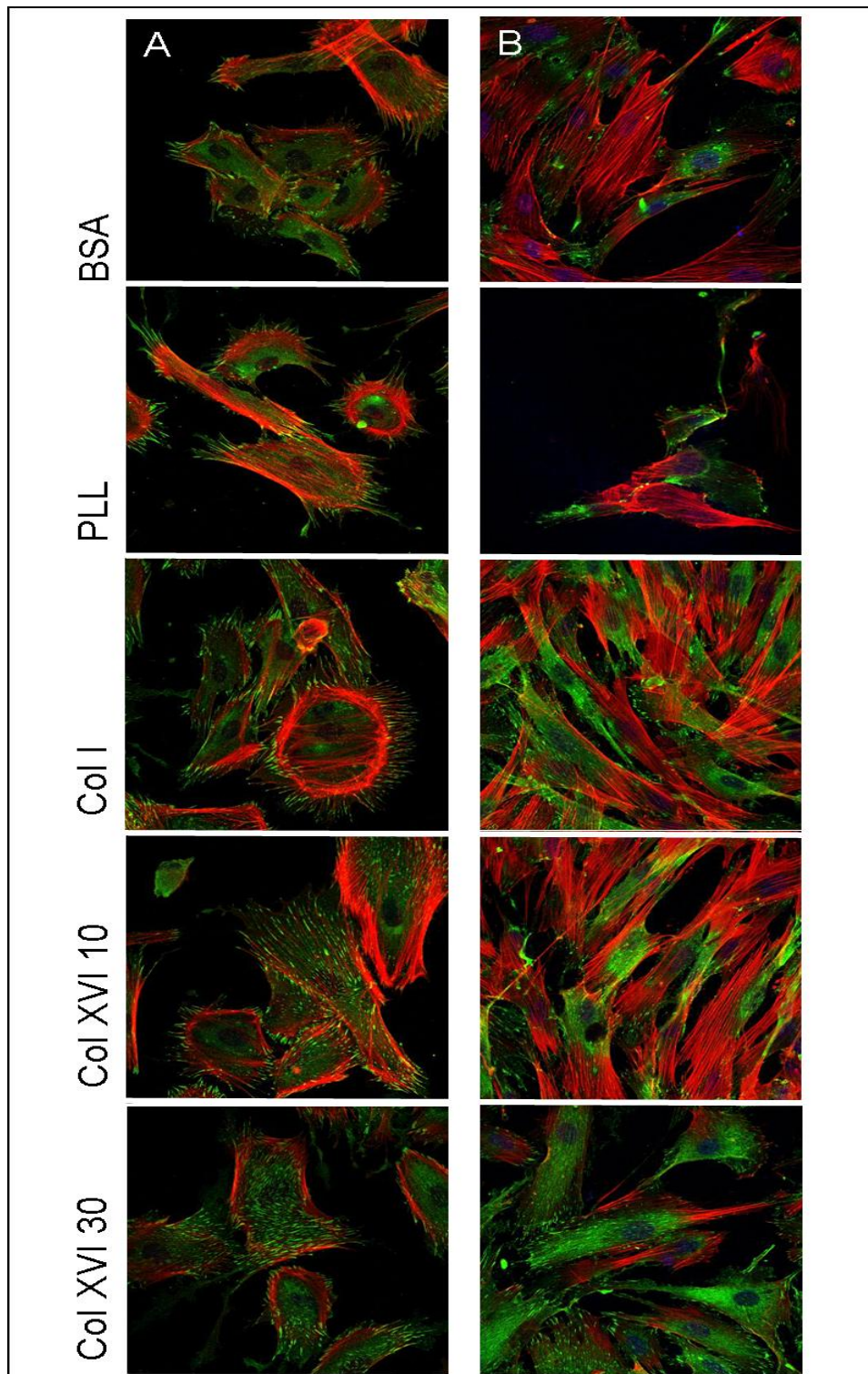
Figure 5-14: Formation of focal contacts – immunofluorescence

Fluorescence stainings for vinculin (green fluorescence) and actin stress fibres (red fluorescence) are depicted for non- (A) and highly inflamed cells (B). Representative images of one patient per inflammation stage are depicted. n = 3 (non inflamed), n = 3 (highly inflamed)
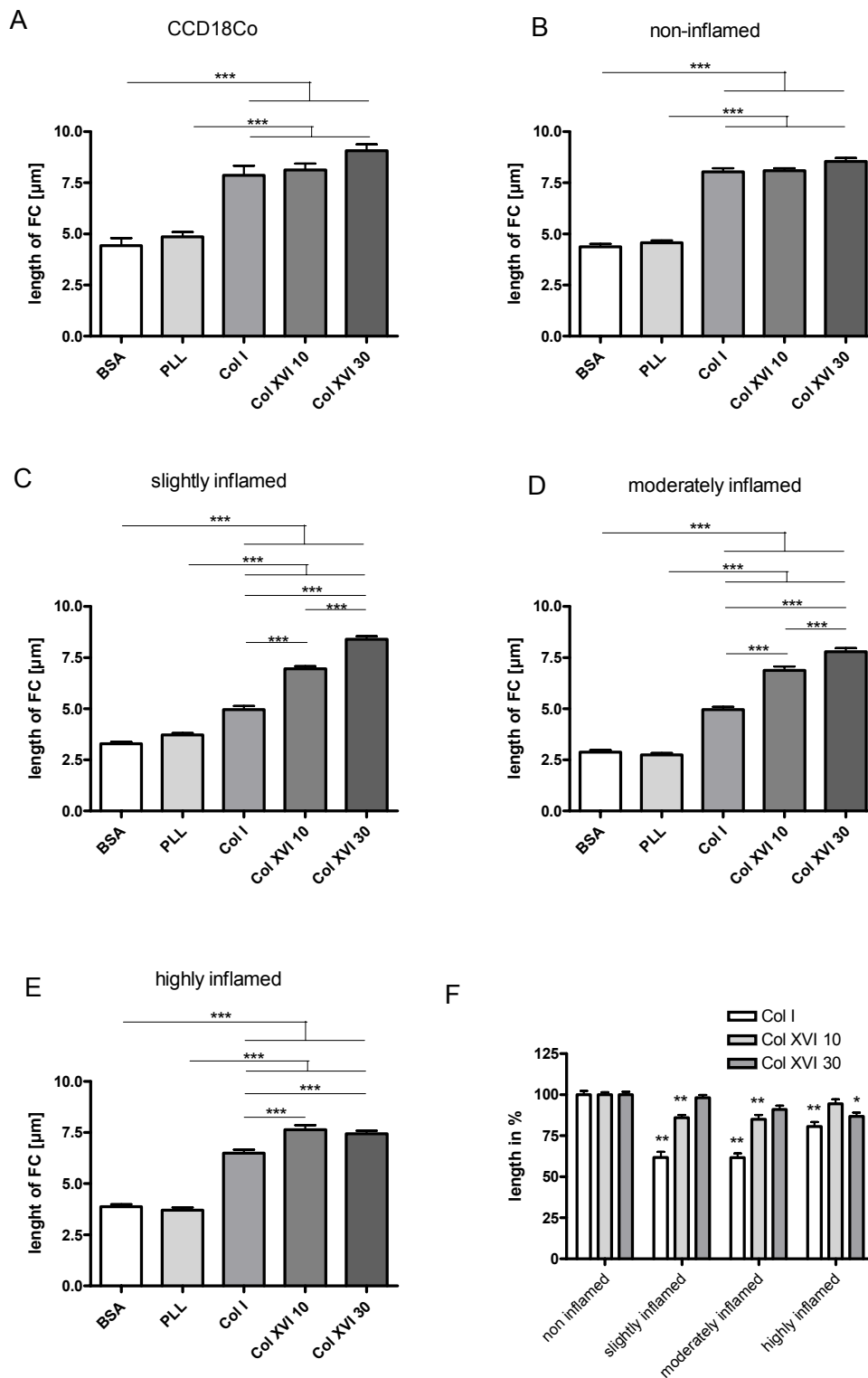
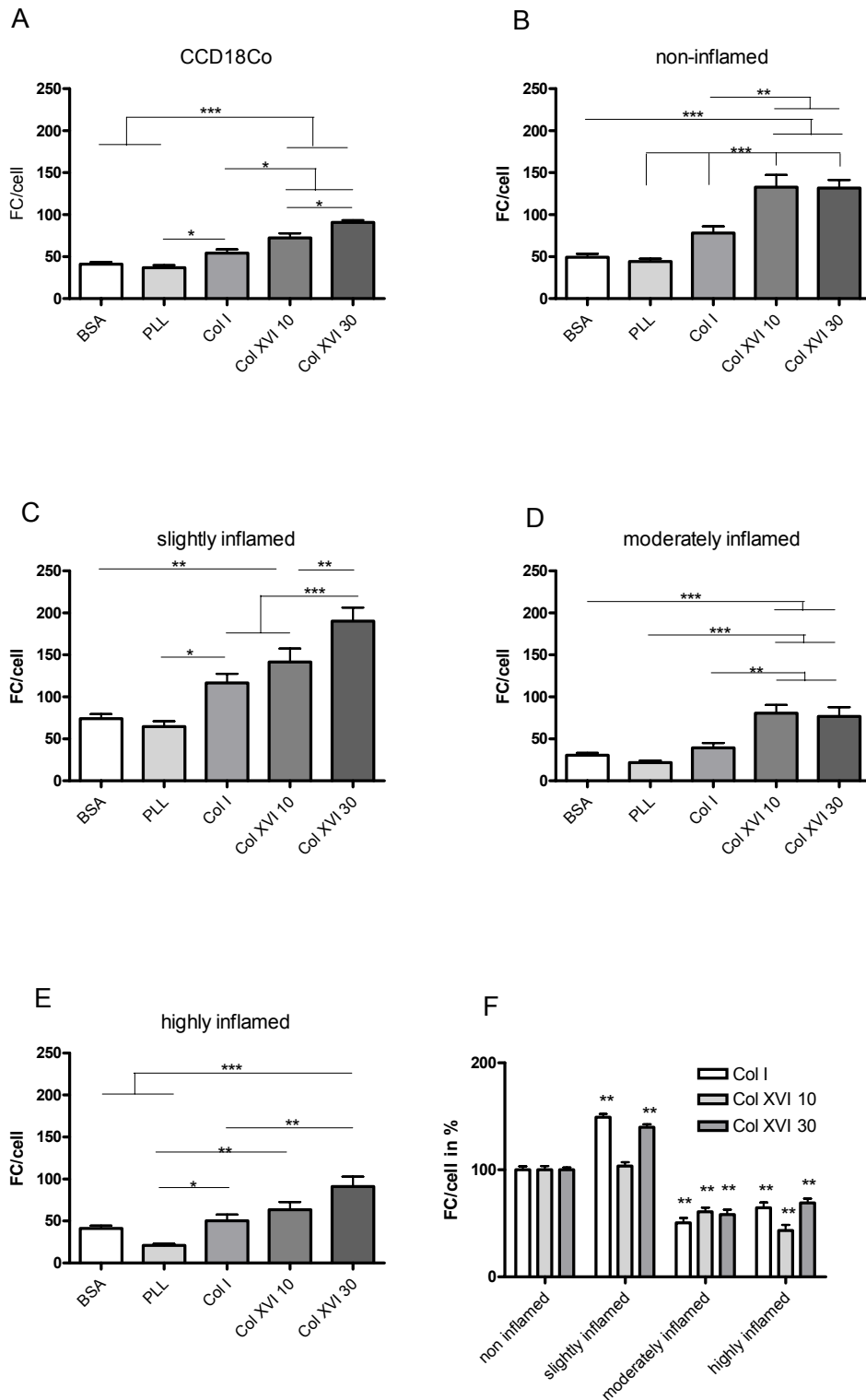**Figure 5-15:** Maturation of focal adhesion contacts

CCD18Co cells and ISEMF were cultured over night on coated glass slides and stained

with ALEXA fluorophores for actin stress fibers (phalloidin coupled antibody) and the

presence of focal adhesion contacts by an anti-vinculin antibody. The length of focal adhesion contacts was evaluated for ISEMF and referred to as the average length. A-E display spectra of focal adhesions length from cells of one inflammation stage on different substrates and F displays tendency of focal adhesion contacts length on collagens I and XVI of the different inflammation stages. n = 5 (non-inflamed), n = 4 (slightly inflamed), n = 4 (moderately inflamed), n = 3 (highly inflamed)

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

5.4.5.7.   Recruitment of α1 integrin into focal adhesion contacts

Double immunofluorescence staining for α1 integrin (red fluorescence) and vinculin (green fluorescence) was compared for all inflammation stages on BSA control, poly-L-lysine, collagen I and collagen XVI. Non-inflamed and highly inflamed cells are depicted in figure 5-16 A and B, respectively. The increased cell spreading on recombinant collagen XVI, demonstrated in scanning electron microscopy in figure 5-11, was confirmed by immunofluorescence staining. On collagen I and collagen XVI, these cells recruited α1 integrin into the focal adhesion contacts. ISEMF on poly-L-lysine, used as control showed focal adhesion contacts, however α1 integrin staining was very weak and remained unchanged in the presence of 0.1 mM $MnCl_2$. Focal adhesion contacts of ISEMF on collagen XVI were not only increased in number like on collagen I but also showed a wider distribution all over the cellular body. On collagen I, focal adhesion contacts were mainly restricted to the cellular periphery. Highly inflamed ISEMF showed increased staining intensity of α1 integrin on collagen I and collagen XVI coatings. Increased staining was also evident in the presence of 0.1 mM $MnCl_2$, which indicates an additional activation of α1 integrin.

A

	vinculin	α1 integrin	merge
PLL			
PLL DMEM+0.1mM Mn²⁺			
Col I			
Col I DMEM+0.1mM Mn²⁺			
Col XVI (10µg/ml)			
Col XVI (10 µg/ml) DMEM+0.1m M Mn²⁺			
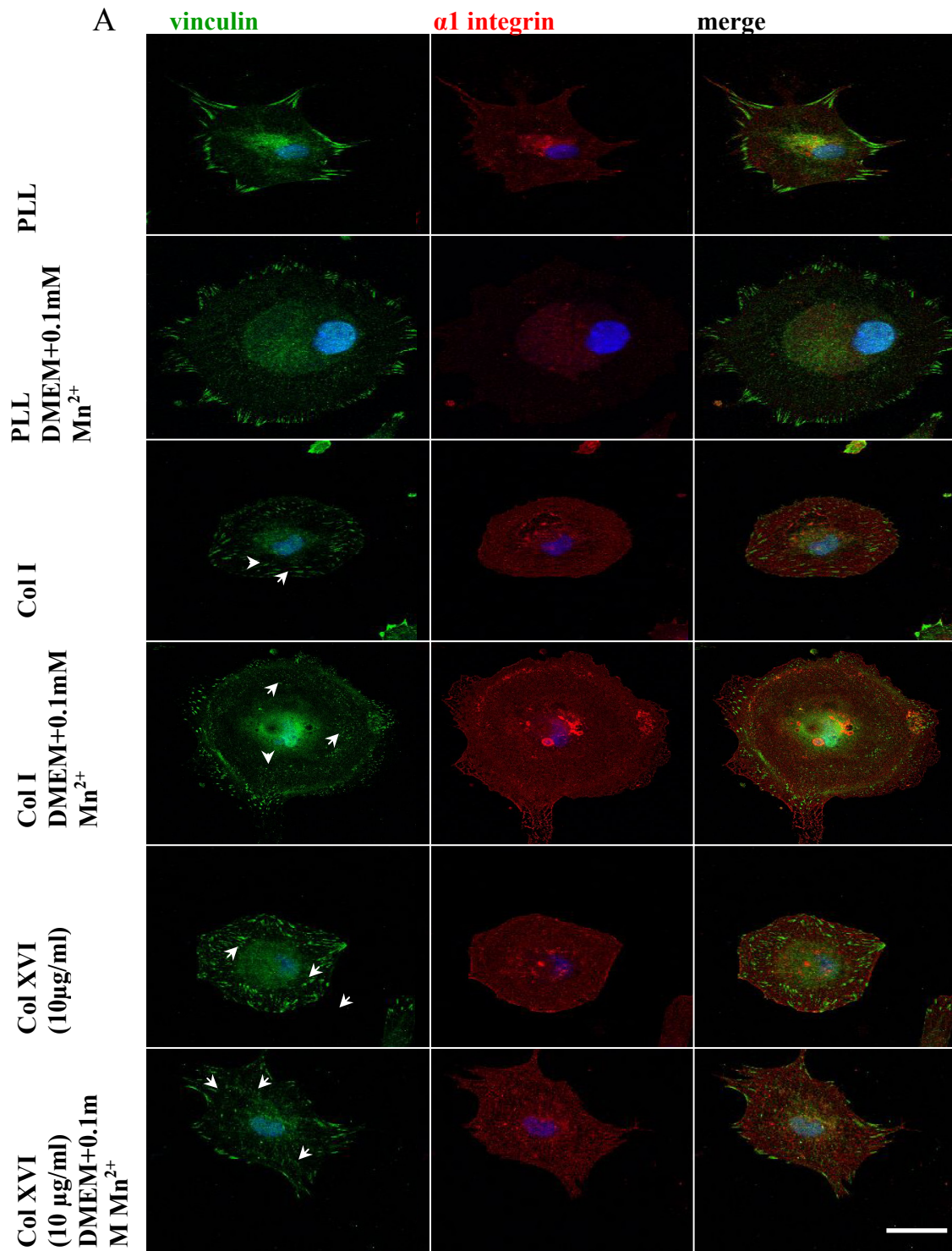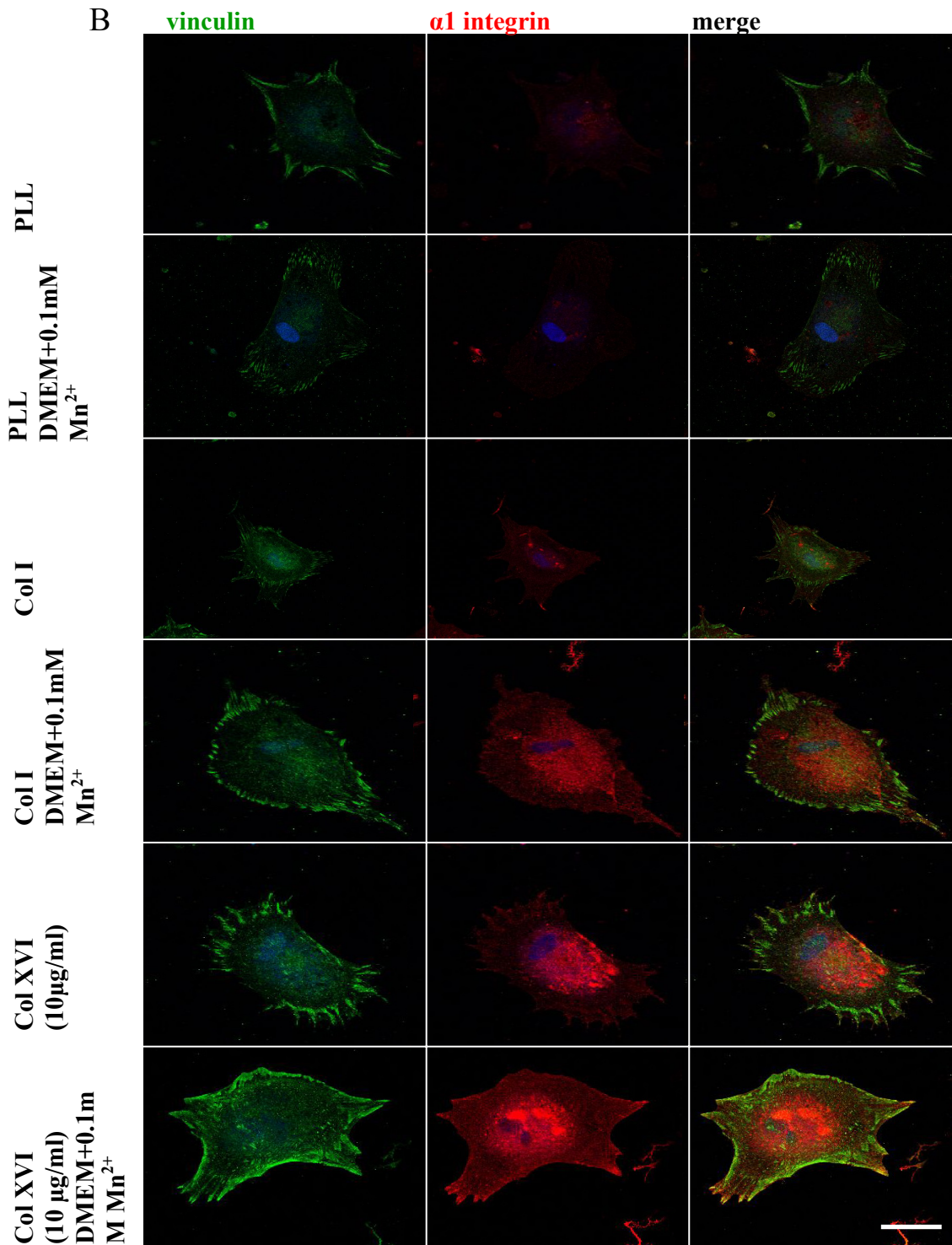
Figure 5-16: Distribution of α1 integrin on the cell surface of ISEMF

ISEMF of different inflammation stages (A: non inflamed, B: highly inflamed) were seeded on coated glass cover plates in the presence and absence of 0.1 mM $MnCl_2$ on poly-L-lysine (PLL), collagen I (Col I) and collagen XVI (Col XVI). After 6 hrs cells were fixed and double-stained with a rabbit antibody against vinculin and a murine antibody against α1 integrin. With laser scanning microscopy α1 integrin (red

fluorescence, middle row) and vinculin (green fluorescence, left row) were visualized with 400 x magnification. Colocalization is depicted in the right row (merge) and appears in yellow color. Bar represents 100 μm.

Part II: Establishment of a retroviral mediated gene silencing model

5.5. Retroviral mediated gene silencing of collagen XVI

5.5.1. Generation of virus particles

The replication incompetent shRNA was stably integrated into the ectropic EcoPack-293 packaging cell line genome. Infectious retroviral particles containing the shRNA were produced and utilized to infect the murine fibroblast cell line NIH3T3. Puromycin restistance enabled selection and enrichment of positive cell clones which had incorporated the plasmid. A *Mlu*I cleavage site located within the shRNA sequence was used as insertion control of the shRNA (data not shown). Positive cell clones were sequenced to determine correct DNA alignment and shRNA sequence (Fig. 5-17).
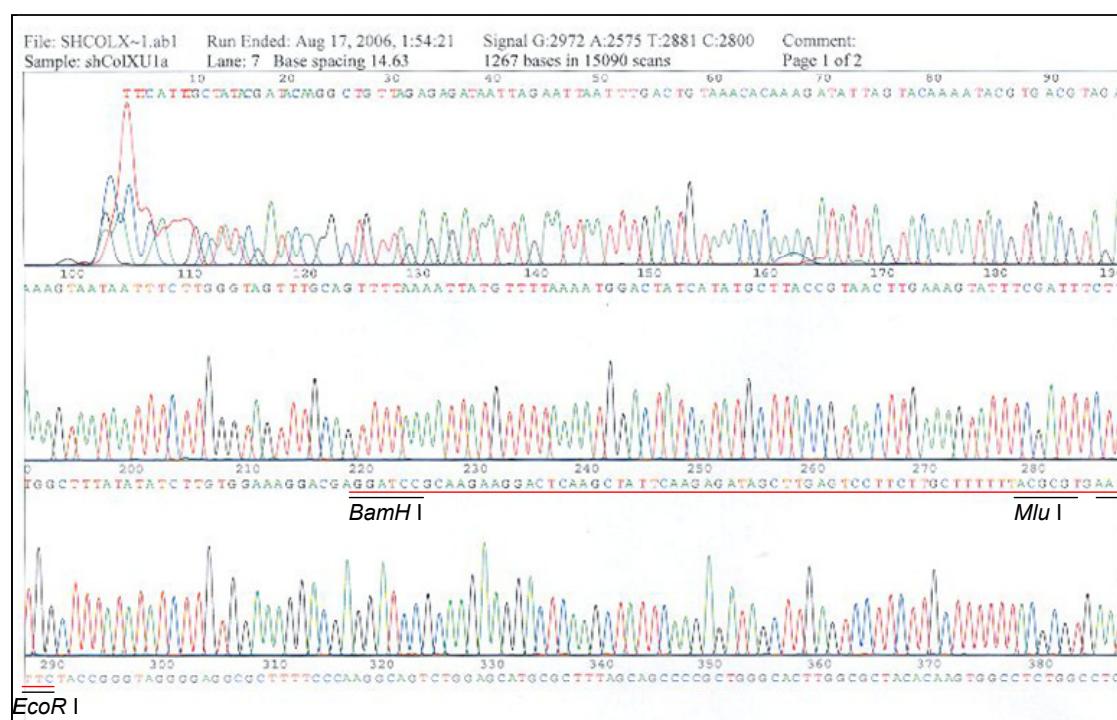
Figure 5-17: Sequence analysis of one shRNA clone (shColXVI1a) showing correct alignment with the plasmid construct. Black lines show restriction overhangs for *BamH*I, *EcoR*I and *Mlu*I integrated in the shColXVI sequence (red line). Sequence outside the marked area represents vector sequence. Further knockdown experiments refer to this shRNA clone.

5.5.2. Controlling gene and protein expression of collagen XVI in NIH3T3 cells after knockdown

Transduction efficiency was evaluated seven days after transduction and antibiotic selection. Gene expression level was determined as absolute cDNA copy numbers using a plasmid standard curve (Fig. 5-18 A). GAPDH gene expression served as control and remained unaffected (data not shown). A knockdown of ~90 % was observed in comparison to the control cells which were transduced with viral particles containing an unrelated luciferase shRNA.

The knockdown was also evaluated on protein level with immunoblotting. An absence of around 100 % could be observed for the full-length collagen XVI chain.
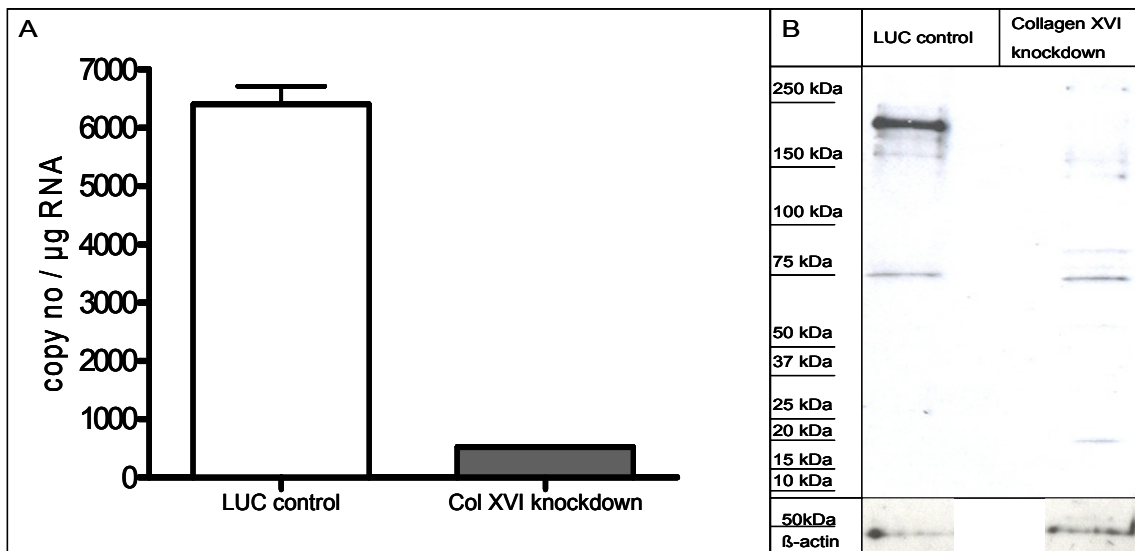
Figure 5-18: Analysis of knockdown efficiency of *Col16a1* was performed at mRNA level with quantitative PCR and shows 91 % lower *Col16a1* gene expression in transduced cells compared to control cells transduced with luciferase shRNA (A). Almost 100 % knockdown was achieved regarding the full-length collagen XVI protein (B, lane 2). ß-actin expression is unaffected which indicates a specific knockdown of collagen XVI. n = 3

5.5.3. Downstream processing of transduced NIH 3T3

Collagen XVI knockdown downstream effects of other expressed proteins were analyzed by 2D-gelelectrophoresis. These results were completed by relative gene expression analysis of these candidate genes. Collagen XVI is actively linked via integrins with the cytoskeleton and a previously performed knockdown of collagen XVI

in human glioma cell lines resulted in a change of actin-related genes (Senner et al., 2008).

5.5.3.1. Identification of differentially expressed proteins by 2D-gelelectrophoresis

For 2D-gelelectrophoresis whole cell lysates of knockdown cells were applied onto separate gels and visualized with Coomassie Blue staining. From all differentially screened spots the two most prominent ones were identified by mass spectrometry. This analysis revealed that peptidyl-prolyl-isomerase A (Fig. 5-19, 1) and macrophage migration inhibitory factor (Fig. 5-19, 2) were absent in collagen XVI knockdown cells.
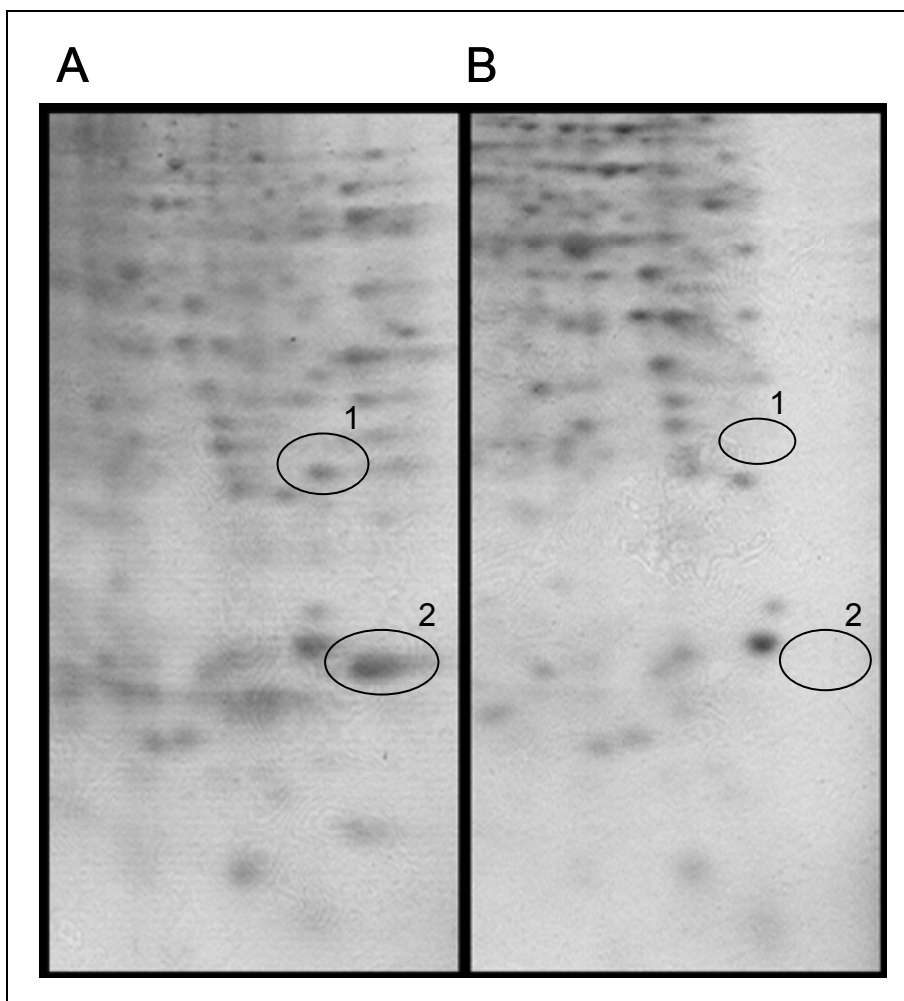
Figure 5-19: Control NIH3T3 cells (A) and crude cell lysates from collagen XVI knockdown (B) were applied on separate gels for 2D-gelelectrophoresis. Among several differentially expressed protein spots two were isolated and further analyzed with MALDI-TOF. Sequence analysis revealed peptidyl-prolyl-isomerase A, PPIA (1) and macrophage migration inhibitory factor, MIF (2) to be absent in knockdown (B). n = 3

5.5.3.2.   Gene and protein expression of MIF and PPIA

Expression of MIF and PPIA was altered in knockdown cells compared to control cells on protein level in 2D-gelelectrophoresis. Gene expression however, revealed a slight up-regulation of both genes determined in relative quantitative PCR.
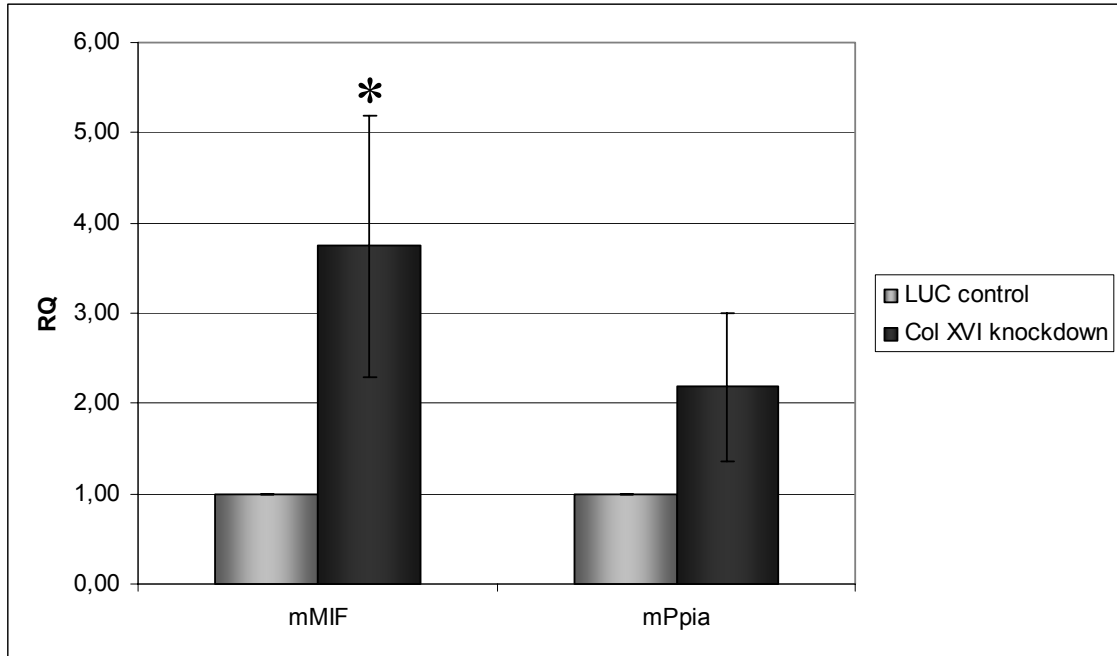
Figure 5-20: Gene expression of MIF and PPIA was investigated with relative quantification ($\Delta\Delta$Ct method) in relation to 18sRNA. Three independent knockdown experiments resulting in a knockdown of 70-90 % showed a slight upregulation of PPIA and MIF.

* $p < 0.05$

The protein expression of MIF and PPIA was determined by immunoblotting of 10 µg RIPA cell lysate. Protein expression was clearly down-regulated in cells lacking collagen XVI (Fig. 5-21).
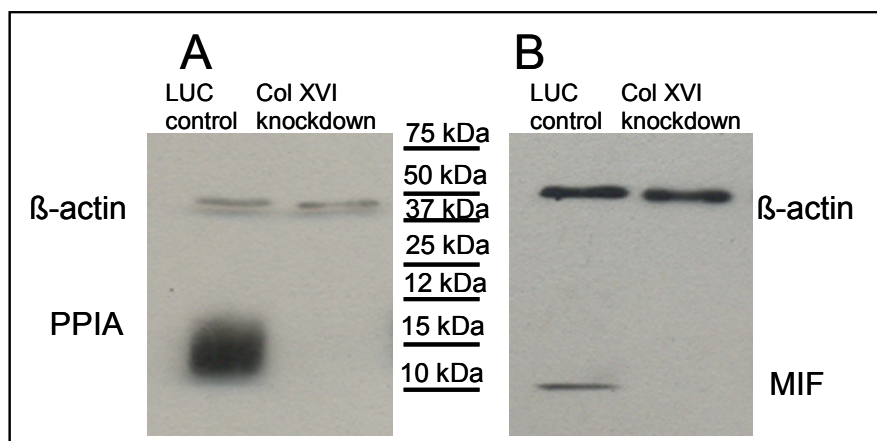
Figure 5-21: Western blot analysis of 10 µg crude cell lysate (RIPA) confirmed results obtained with 2D-gelelectrophoresis. Here, PPIA and MIF showed a lower expression on protein level. ß-actin served as loading control. n = 3

5.5.3.3.   Functional analysis of collagen XVI silenced NIH3T3
5.5.3.3.1.   Adhesion capacity

Adhesion of control and knockdown cells was determined after 30 min (Fig. 5-22 A), 90 min (Fig. 5-22 B), and 18 hrs (Fig. 5-22 C) on several coatings (1 g/ml BSA; PLL: 5 µg/ml poly-L-lysine; Col I: 10 µg/ml collagen I; Col XVI 10: 10 µg/ml collagen XVI; Col XVI 30: 30 µg/ml collagen XVI). After 30/90 min, profound increased adhesion was observed for luciferase control compared to knockdown cell. After 90 min, adhesion differences due to the coating material are no longer evident. On all coatings a reduced adhesive capacity was observed for NIH3T3 cells with a collagen XVI knockdown. After 18 hrs no adhesion differences were detected.
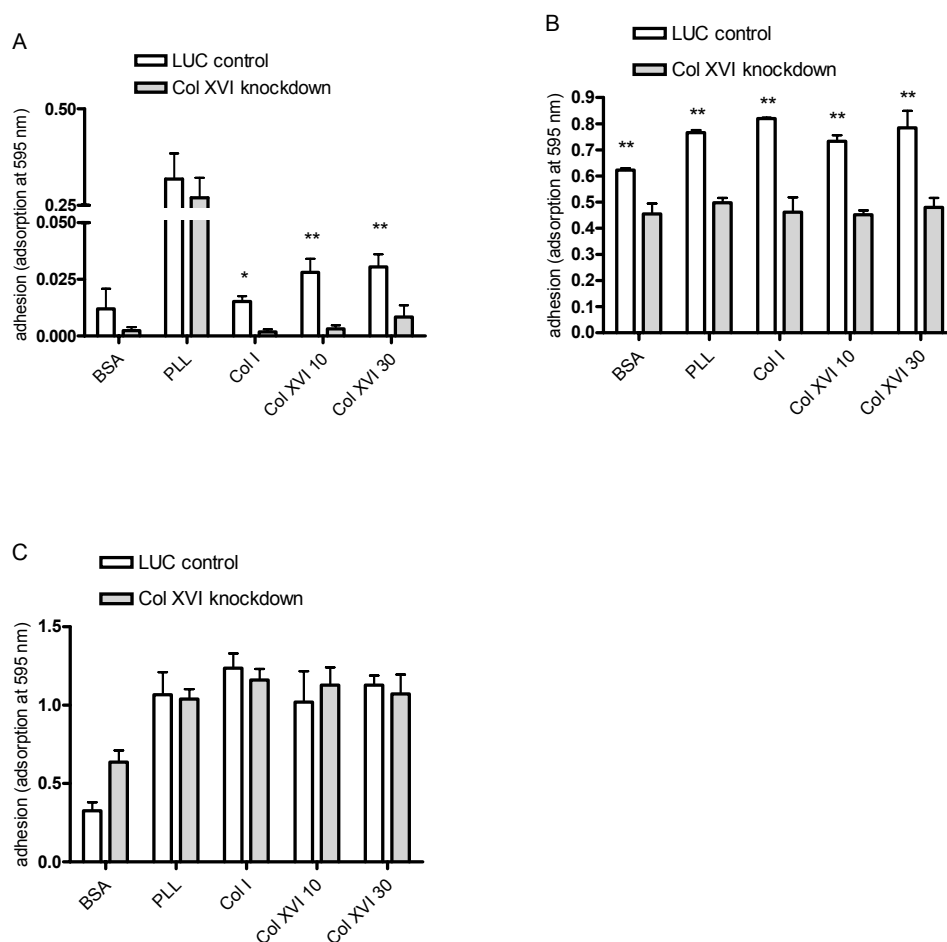
Figure 5-22: Adhesion assay on different coating substrates with collagen XVI silenced NIH3T3 cells compared to the luciferase control.

The adhesion assay was performed with three independent knockdown cell batches ranging from 70 % – 90 % collagen XVI knockdown. * p<0.05; ** p<0.01

5.5.3.3.2. Proliferation properties

Figure 5-23 displays data obtained from assaying proliferation (EZ4U) of luciferase transfected and collagen XVI knockdown cells. Proliferation assay was performed using 1 % (w/v) BSA, poly-L-lysine (5 µg/ml), collagen I (10 µg/ml), and two different concentrations of collagen XVI (10 µg/ml and 30 µg/ml). Cells did not show differences in total cell number with respect to the culture substrates, however, NIH3T3 with reduced collagen XVI expression had lower cell numbers and thus were presumably restricted in their proliferation capacity on all coatings.
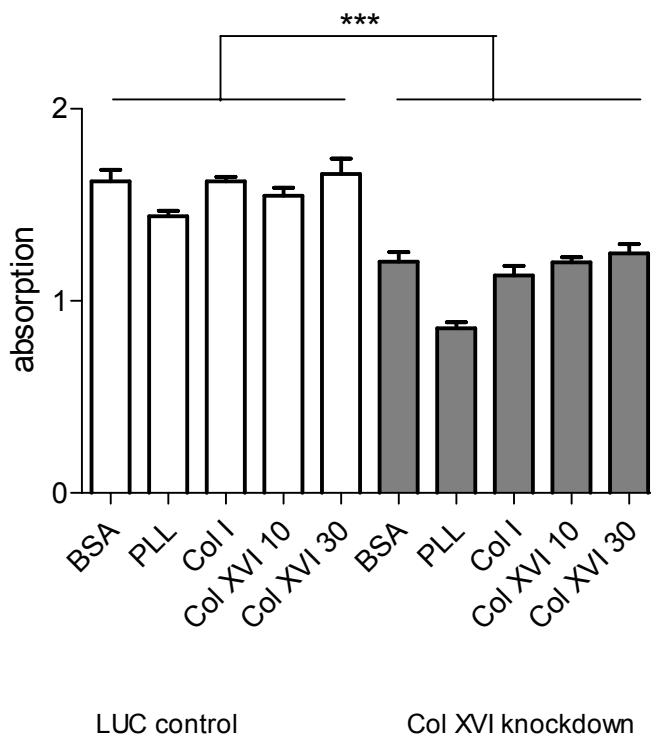
Figure 5-23: Proliferation properties of collagen XVI knockdown cells and luciferase control.

Proliferation assay (EZ4U) was performed with collagen XVI knockdown cells and NIH3T3 transfected with the luciferase control seeded on coated well plates. Data of three independent knockdown experiments were combined. *** p<0.001

5.5.3.3.3. Migratory behaviour

Scratch assay of collagen XVI knockdown cells and luciferase control cells on tissue culture surface revealed no differences in migration (data not shown).

6. Discussion

"It is better to know some of the questions than all of the answers." James Thurber

Part I: The role of collagen XVI in pathological disorders

6.1. Recombinant expression of collagen XVI

Collagen XVI is a minor component of specialized tissues like skin or cartilage and therefore, it is difficult to isolate larger amounts of the authentic protein from tissue samples. Isolation methods that are usually applied for fibrillar collagens are not suitable for FACIT collagens like collagen XVI (Deyl and Miksik, 2000). Pepsin digestion performed in this method destroys non-collagenous proteins and non-collagenous domains thus leaving a fragmented collagen XVI (Grassel et al., 1996). Formerly established protocols for recombinant collagen XVI production in a HEK 293 EBNA expression system have been successfully applied in this work (Kassner et al., 2004). The HEK 293 EBNA cell line expresses the Epstein-Barr-Virus (EBV) Nuclear Antigen 1 (*EBNA1*). Therefore, the cells are enabled to keep plasmids containing a resistence gene in addition to an EBV-replication origin stably integrated episomal. The utilized pCEP Pu vector contains a BM40 signal peptide for increased secretion of proteins and a C-terminal StrepII-Tag which allows affinity purification of non-denatured recombinant protein.

Expression rates of collagen XVI were increased from documented 1.5 mg up to 3.0 mg recombinant protein per liter conditioned medium purified by affinity chromatography with a Strep-Tactin-Superflow column. Culture of collagen XVI expressing HEK 293 EBNA cells in plastic roller flasks instead of glass roller flasks allowed longer cultivation duration, an up-scale in culture surface from 100 % up to 240 %, and thus a doubling in protein yield.

The molecular weight of recombinant collagen XVI was determined by electrophoretical mobility (Kassner et al., 2004). The full-length alpha1 chain (213 kDa) plus proteolytically cleaved fragments were identified: a 182 kDa polypeptide lacking the NC11 domain, a 78 kDa fragment containing two C-terminal collagenous domains (COL1 and COL2), and finally a 133 kDa polypeptide, presumably lacking C-terminal domains. All of these processed proteins were detected after affinity purification.

6.2. Collagen XVI in Crohn's disease

6.2.1. Molecular distribution and cellular expression of collagen XVI

During the course of Crohn's disease the composition of the intestinal ECM has an immense impact on the behaviour of therein occurring cells and progression of the disease. Data on excessive collagen matrix production in CD patients has been either focused on fibrillar collagens as collagen I, III or on collagens associated to basement membranes (collagen IV). Cellular origin and distribution of FACIT collagens and their contribution to matrix alterations in intestinal inflammatory processes have not been addressed yet. The functional and structural diversity of extracellular matrices is determined by individual macromolecules and the aggregates they form. Although, quantitatively major matrix molecules as fibrillar collagens can occur ubiquitously, their organization varies tissue specifically due to their association with specific sets of minor components, i.e. FACIT-collagens. They are part of the fibrillar periphery constituting the interface between extrafibrillar matrix and the fibrillar body. These collagens can attach adapter molecules as matrilins and COMP capable of interconnecting macromolecular networks and thus mediating interactions between fibrillar aggregates and the extrafibrillar matrix including cells (Budde et al., 2005; Kassner et al., 2003). Therefore, an intact peripheral composition of fibrils is a prerequisite for the integrity and stability of the whole ECM. Presence and distribution of FACIT-collagens in skin, cornea and cartilage have been well documented, however, information about localization, metabolism, and cellular source of FACIT-collagens in the bowel wall is unavailable. Our histological studies demonstrated deposition of collagen XVI in the normal, non-inflamed and inflamed, due to fibrosis expanded, submucosa and lamina propria of the strictured bowel. Both, epithelial cells and subepithelial myofibroblasts which are localized subjacent to the basement membrane and in close vicinity of the basal surface of the epithelial cell layer express collagen XVI with a prominent deposition of collagen XVI into the matrix of the epithelial crypts. This particular distribution is inverse to the dermal situation where collagen XVI is localized in defined zones adjacent to basement membranes preferentially on the dermal side of the dermo-epidermal junction (DEJ) even though expressed by keratinocytes (Grassel et al., 1999). We suggest collagen XVI to contribute to stabilization and maintenance of basement membranes in the normal bowel wall as it was proposed for the DEJ in skin. It is predominantly expessed by intestinal myofibroblasts of the lamina propria and epithelial cells of the crypts. Increased gene and protein expression in ISEMF isolated

from inflamed CD tissue biopsies indicates a distinct role of collagen XVI during progression and/or maintenance of inflammation.

6.2.2. Role of collagen XVI in adhesion, migration, and proliferation potential of ISEMF

Adhesion properties influenced by collagen XVI

The first phase in integrin-mediated signal transduction is the formation of focal adhesion contacts. Our study on the integrin expression pattern of differently inflamed ISEMF revealed increasing α1 integrin expression concomitant to elevated collagen XVI gene and protein expression. Four collagen-binding I domain-containing integrins are currently known: α1β1, α2β1, α10β1 and α11β1 (Gullberg and Lundgren-Akerlund, 2002). For the integrins α1 and α2 an interaction with collagen XVI has been determined and a binding site for α1 integrin was identified in the C-terminal domain of collagen XVI (Eble et al., 2006). α1β1 is proposed as sole integrin utilized by contracting myofibroblasts *in vivo*. Although α2β1 is capable of mediating contraction, its expression by myofibroblasts is supposed to occur largely, if not exclusively, in response to cell culture (Racine-Samson et al., 1997). α2ß1 integrin has been shown to be dispensable for fibroblast adhesion (Zhang et al., 2006). Collagen-binding integrins are co-expressed by fibroblasts, however, they may not act simultaneously. α2ß1 integrin is important in the very early steps of fibroblast adhesion to collagen I. In later stages of adhesion other receptors can completely or partially compensate for the lack of α2ß1 integrin (Zhang et al., 2006). α1ß1 integrin is responsible for fibroblast adhesion to collagen I, collagen IV and laminin, as well as to collagen XVI (Eble et al., 2006; Zhang et al., 2006). Cell-free protein binding assays revealed a stronger interaction of α1ß1 integrin with collagen XVI than α2ß1 integrin. Dependent on the cell type, adhesion is facilitated by several integrins. A rat glioma cell line (RuGli) adhered via α1ß1 integrin to collagen XVI (Eble et al., 2006), whereas human fibrosarcoma cells showed mainly interaction via predominantly expressed α2ß1 to collagens in general (Perret et al., 2003; Vandenberg et al., 1991).

Integrin-collagen binding results in mechanical anchorage of cells and signals are transmitted from the ECM via integrins into the cells. The formation of focal adhesion contacts is the primary step in integrin-mediated signal transduction. Our immunofluorescence studies on α1 integrin showed a recruitment of this integrin into

the supramolecular structures of focal adhesion contacts. To visualize focal adhesion contacts, we used vinculin, a protein present in focal adhesion plaques. Focal adhesion contacts of ISEMF on collagen XVI contained α1 integrin and vinculin and were increased in number and length. Inflamed ISEMF revealed generally increased α1 integrin levels and also a recruitment of this integrin into focal adhesion contacts all over the cellular body when seeded on collagen XVI. Cells spread on collagen XVI similar to collagen I, however, have longer streak-like focal adhesion contacts. Together with increased collagen XVI gene and protein expression in ISEMF from inflamed tissue it suggests that collagen XVI is a physiological substrate for myofibroblasts *in vivo* and may act as agonist inducing an integrin-mediated signal transduction resulting in increased cell spreading and alterations in cell morphology. ISEMF that have been adhered on collagen XVI developed an extensive cell spreading compared to collagen I where cells remained more roundish. Analysis of development and maturation of focal adhesion contacts formed by ISEMF revealed elevated formation on collagen XVI as culture substrate compared to collagen I and poly-L-lysine. The maturation of focal adhesion contacts was also augmented by collagen XVI. After 24 hrs ISEMF revealed longer focal adhesion contacts when seeded on collagen XVI compared to poly-L-lysine, however, not significantly different from those formed on collagen I. Nevertheless, our adhesion assays revealed no significant changes in adhesion capacity of ISEMF with respect to increasing inflammation on collagen XVI, whereas ISEMF from non-inflamed tissue biopsies adhere less to collagen XVI compared to collagen I. This might be due to a differential integrin surface expression profile or clustering of ISEMF in inflamed regions of the intestinal wall. We demontstrated that ISEMF from normal colon tissue biopsies express less α1 integrin as do cells from inflamed areas. This integrin has a high affinity recognition site in the C-terminal region of collagen XVI and binds with profound higher affinity to collagen XVI as does the α2 integrin subunit (Eble et al., 2006).

Fibrotic tissue reveals extensive cell-matrix contacts that are absent from normal connective tissue (Eyden, 2005). Our data implicate that collagen XVI is a preferred substrate for the formation of focal adhesion contacts and therefore promotes more stable and prolonged adhesion of ISEMF to the underlying matrix. This observation is supported by the distribution pattern of focal adhesion contacts. On collagen XVI as substrate focal adhesion contacts are also found in the centre of the cell, whereas on poly-L-lysine focal adhesion points are mainly restricted to the cellular periphery.

Collagen XVI seems to provide more stiffness resulting in higher tension that immobilizes cells. Recently, we have shown that collagen XVI promotes adhesion of glioblastoma cell lines thus presumably playing a role in regulation of adhesive properties of tumor cells (Senner et al., 2008). Possibly, increased accumulation of collagen XVI in inflamed colon tissue would help to promote a pathological continuance of ISEMF by keeping the cells at the inflammation site. It is conceivable that collagen XVI is a preferred substrate for formation of focal adhesion contacts and thereafter extensive cell spreading since it might be easier to contact by the cells due to its exposed peripheral location on fibrillar structures compared to collagen I which forms the body of fibrils.

Migration and proliferation properties influenced by collagen XVI

In normal tissue repair fibroblasts migrate into the open wound where they synthesize and remodel the ECM to close the wound. Fibrosis occurs by the failure to terminate the physiological tissue remodelling program. The crucial point in tissue repair is the ability of fibroblasts to attach to ECM followed by cell migration and ECM contraction (Hinz and Gabbiani, 2003). Adhesion to ECM is facilitated by integrins who transmit extracellular signals through focal adhesion kinase (FAK). FAK is present in focal adhesions and is phosphorylated after intergrin-mediated cell attachment (Parsons, 2003). Fibrotic cells are characterized by activated adhesion and adhesive signalling (Chen et al., 2005; Desmouliere et al., 2005; Mimura et al., 2005). Adhesion is not only a process engaging attachment of fibroblasts to ECM, however, also results in downstream expression of genes involved in tissue remodelling and repair. Therefore, fibroblast attachment is actively interacting with a promotion in tissue repair processes and may induce the expression and secretion of components of the ECM (Kennedy et al., 2008). During culture of fibroblasts ECM is synthesized and deposited. Differences in ECM production of fibrotic or non-fibrotic cells may significantly alter ECM composition and pro-fibrotic gene expression in a feed-back loop (Shi-Wen et al., 2007). Attachment to the ECM regulates proliferation and migration of cells (Schwartz and Assoian, 2001), however collagen XVI as substrate did neither alter proliferation nor migration rates of ISEMF. Collagen XVI rather seems to immobilize initially adhered ISEMF to the normal colon wall and to a higher extent at the inflammation site in late stages of the inflammation process than to influence proliferation or migration properties. Focal adhesion contacts are found all over the cell which is characteristic for

highly motile focal adhesion contacts in stationary cells (Smilenov et al., 1999). Although, we have not found a proliferation or migration promoting effect of pure collagen XVI in our monolayer migration assays, collagen XVI may act as migration promoting substance in combination with other factors e.g. in allocation of three dimensional structures through interconnection to other structural proteins in a mucosal specific ECM. The proliferation experiments refer to total cell number, indirectly determined by enzyme activity. As we could exclude any effect of culture substrates on cell viability, we assume a direct relation of total cell number to proliferation.

Different collagens co-assemble into heterotypic fibrils. The relative composition and the amounts of these collagens define the structure and organization of the extracellular matrix network and determine biomechanical properties of these tissues. Members of the FACIT collagens are multi-domain molecules that are associated with collagen fibrils to form more complex alloys. The regulated expression of these peripheral molecules represents the molecular basis of the structural diversity of connective tissue. FACIT collagens serve as molecular bridges that are important for the organization and stability of extracellular matrices. Therefore, abnormalities in the expression of FACIT collagens may also contribute to pathological changes in several connective tissues (Akagi et al., 1999; Senner et al., 2008).

Summary and conclusion

Recombinant expression of collagen XVI was reproducible and allowed a variety of experimental set-ups with respect to investigations of pathological disorders and functional studies on collagen XVI.

Supramolecular structures which exhibit a variety of compositions of their components support mechanical properties of specialized tissues. The deposition of collagen XVI into the lamina propria and submucosa by myofibroblasts and epithelial cells of the human bowel wall is inverse to the dermal distribution. Collagen XVI is associated to fibrillin-1 containing microfibrils in the human skin and to D-periodically banded cartilage fibrils, whereas in the human colon a specific binding partner is still unknown. Collagen XVI presumably acts as adapter molecule by interconnecting marcromolecular structures. The distribution pattern of collagen XVI in the submucosa and lamina propria may support the motility of fibrous tissue compartments in the human bowel wall. However, with increasing inflammation and beginning fibrosis the homeostasis and peristaltic movement of the bowel is disturbed. In these pathological situations

observed in CD, collagen XVI gene and protein expression is up-regulated. The intestinal myofibroblast is the key player in chronic bowel wall inflammation processes. These cells mediate stricture formation and are responsible for excessive collagen matrix production and deposition. Collagen XVI is produced by ISEMF in the normal intestine and its synthesis is increased in the inflamed bowel wall. Collagen XVI promotes cell spreading and formation of focal adhesion contacts, but has no influence on proliferation and migration of ISEMF in our experimental set up. ISEMF develop increased numbers of focal adhesion contacts concomitant with α1 integrin expression. Focal adhesion contacts also displayed an increase in length on collagen XVI and that way promoting extensive spreading of the cells. This behaviour presumably adds to the maintenance of cells in the inflamed intestinal regions and thus promotes fibrotic responses of the tissue and prolongs further disturbances of the delicate homeostasis between cells and surrounding ECM. We suggest that collagen XVI can alter force transmission and ECM remodelling by changing the mechanical properties of the ECM.

Part II: Establishment of a retroviral mediated gene silencing model

6.3. Gene silencing of collagen XVI in murine NIH3T3 fibroblasts

6.3.1. Downstream gene and protein expression

2D-gelelectrophoresis of knockdown NIH3T3 cell lysates showed differentially expressed proteins due to the collagen XVI knockdown compared to the control. The housekeeping protein ß-actin remained unaffected indicating collagen XVI gene silencing to be specific and not because of RNAi induced unspecific silencing or viral interferon mediated off-target mechanism. Differentially expressed proteins in collagen XVI silenced cells were identified as macrophage migration inhibitory factor (MIF) and prolyl-peptidyl-cis-trans-isomerase A (PPIA). PPIA is is an abundant, ubiquitously expressed protein that influences the conformation of proteins in cells (Colgan et al., 2000). It catalyzes cis-trans-interconversions of peptides and is involved in general physiological processes like the maintenance of protein structure (Schiene and Fischer, 2000). PPIA is commonly used in gene expression analysis as housekeeping gene appearing less regulated than GAPDH (Feroze-Merzoug et al., 2002). However, in murine NIH3T3 fibroblasts silenced for collagen XVI its expression was dramatically decreased. MIF is a proinflammatory autocrine mediator which influences proliferative and oncogenic processes (Liao et al., 2003). As a central regulator of innate immunity

and inflammation it is associated with tissue injury (Hardman *et al.*, 2005) and was down-regulated in collagen XVI silenced NIH3T3 fibroblasts.

6.3.2. Functional consequences of collagen XVI knockdown

Proliferation and adhesion assays were performed for knockdown NIH3T3 fibroblasts and controls and revealed a reduced adhesion and proliferation capacity of cells lacking collagen XVI. However, adhesive and proliferative potential were not affected by collagen I or collagen XVI serving as culture substrates.

Collagen XVI silenced fibroblasts show a generally reduced proliferation capacity which is not related to the external ECM-stimulus in parallel to a decrease in MIF protein expression. MIF is known to be secreted in a PKC-dependent way as a consequence of cell adhesion to the extracellular matrix and plays a role in integrin-mediated signalling to sustained MAP kinase, cyclin D1 expression, and cell cycle progression (Liao et al., 2003). MIF was initially described as soluble factor in a culture medium of activated T-cells (Bloom and Bennett, 1966); however, without elucidating the accurate biological function. The anterior pituitary gland relases MIF as a hormone in endotoxin shock (Bernhagen et al., 1993; Nishino et al., 1995). It is also produced as a proinflammatory cytokine and glucocorticoid-induced immonomodulator by macrophages in response to a variety of inflammatory stimuli (Calandra et al., 1994). In synovial fibroblasts of rheumatoid arthritis patients MIF up-regulates IL-8 gene expression. This indicates a role in the migration of inflammatory cells into the synovium of rheumatoid joints via the IL-8 induction (Onodera et al., 2004). MIF is a critical regulator of cell functions such as gene expression, proliferation or apoptosis. It is reported that MIF activates the ERK pathway involving phosphorylation and activation of Raf-1, MEK, ERK-1/2 and Elk-1 (Swant et al., 2005). A study on MIF knockout mice revealed an accelerating effect of MIF in cutanous wound healing (Zhao et al., 2005). We suggest a secondary effect of the collagen XVI knockdown on cellular proliferation mediated by a reduction of MIF protein expression. In contrast to protein expression gene expression of MIF was not down-regulated by collagen XVI silencing. In prostate cancer cells MIF showed increased levels of gene expression and a higher mRNA stability compared to prostate epithelial cells (Meyer-Siegler, 2000). We assume a delayed effect on MIF gene expression due to longer mRNA half-life and therefore a discrepancy between gene and protein expression. The lack of collagen XVI resulting in a reduction of MIF protein expression may therefore act in a negative feedback loop on

cellular proliferation. After 90 min of adhesion the rates of knockdown cells remained below those of the luciferase vector control. However, reduced proliferation capacity is not a consequence of a lower number of adhered cells as demonstrated for 18 hrs of adhesion. The knockdown of collagen XVI may influence cellular mechanisms like proliferation. However, the type of culture substrate did not influence this behaviour significantly.

Like MIF, the prolylpeptidyl-cis-trans isomerise A (PPIA) was down-regulated in collagen XVI knockdown fibroblasts. PPIA catalyzes cis/trans isomerization of the peptide bond preceding the proline in a polypeptide chain. This process is important in de novo protein folding and in isomerization of native proteins (Endlich et al., 2006). Changes in isomerase activity can alter the transactivation potential, protein stability or intra-cellular localization of proteins. Disturbed cell proliferation and tumorigenesis are linked with these processes (Shaw, 2007). However, a direct relation of PPIA with collagen XVI has to be further elucidated.

Summary and conclusion

Retroviral mediated gene silencing together with 2-D-gelelectrophoresis provides potent tools for downstream gene-function analysis. The knockdown of collagen XVI established in NIH3T3 cells can be transferred to any dividing cell type. Therefore, a large variety of functional testing is possible. For the NIH3T3 cells the specific collagen XVI silencing results in a generally reduced proliferation and adhesion capcity accompanied by down-regulation of MIF and PPIA protein expression. The reduction of PPIA could influence correct protein folding, however, remains to be further investigated. The decrease in MIF may indicate an indirect effect of collagen XVI knockdown on cellular proliferation. Initial adhesion of collagen XVI silenced cells is disturbed and irrespective of the coating substrate.

7. Affixes

7.1 Bacterial cloning vectors
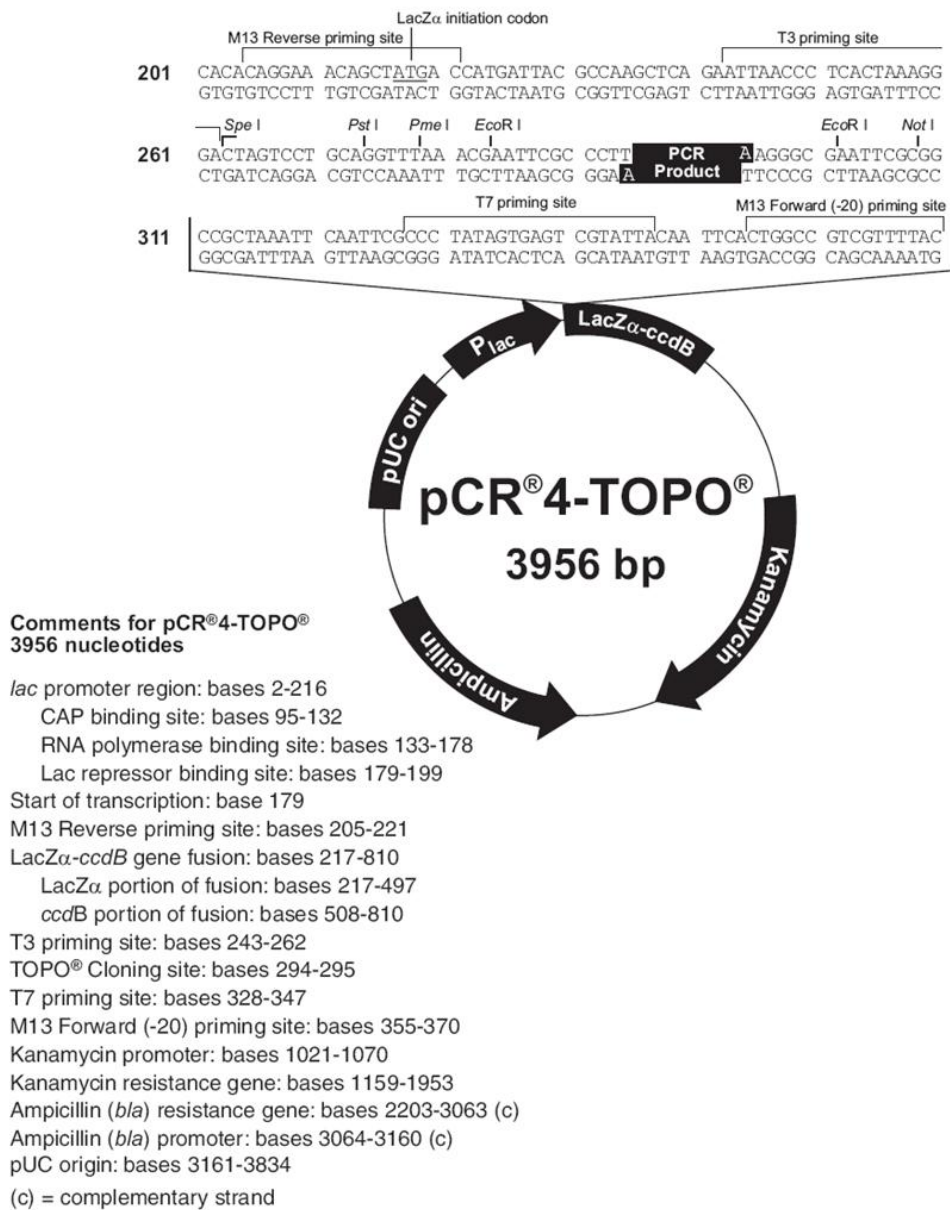
Figure 7-1: Cloning vector pCR®4-TOPO (ampicillin and kanamycin resistance, Invitrogen, UK)
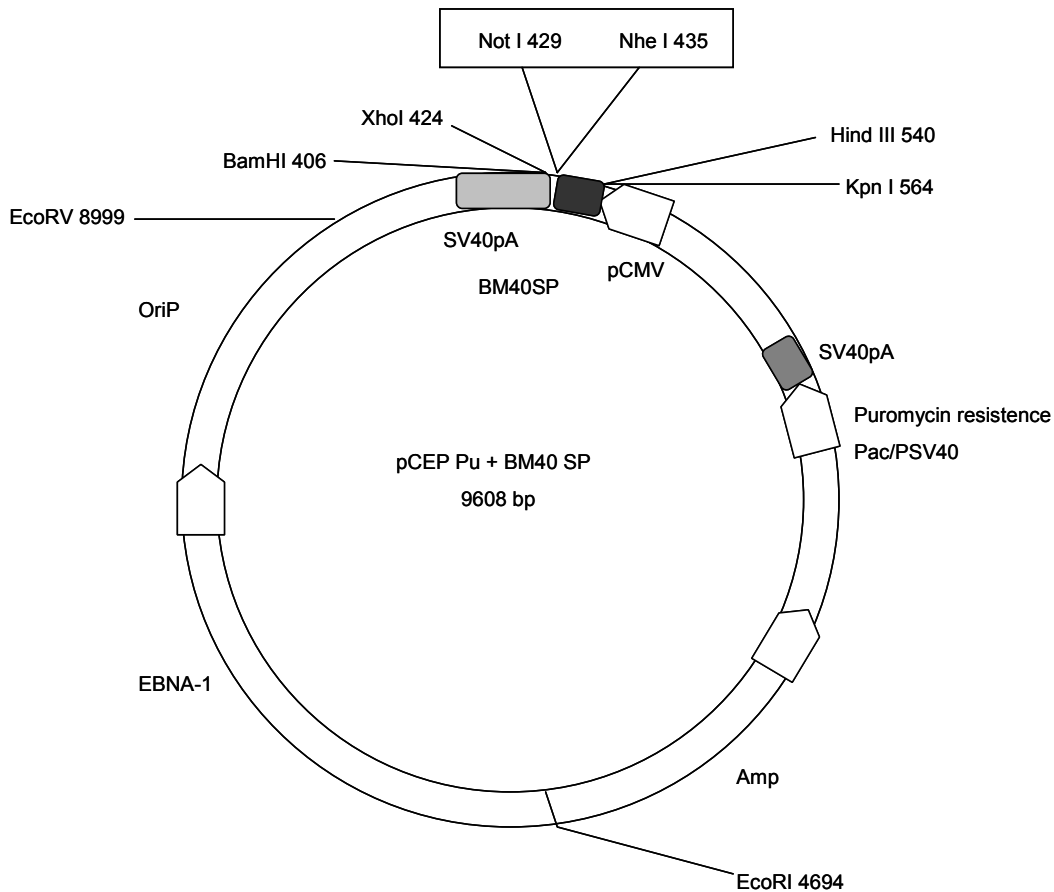
Figure 7-2: Expression vector pCEP PU BM40SP

Not I and Nhe I within the MCS are framed. The black area refers to the coding sequence of the BM40 signal peptide (BM40SP). The vector contains an ampicillin resistance for selection in prokaryotes and a puromycin resistance for selection in mammalian cells.

This vector is based on a no longer commercially available expression vector pCEP Pu from Invitrogen, UK. The vector has been modified by insertion of a BM40 signal peptide and several tag sequences (Kohfeldt et al., 1996; Kohfeldt et al., 1997; Smyth et al., 2000) and recombinant expression of collagen XVI with this vector has been established previously (Kassner et al., 2004).
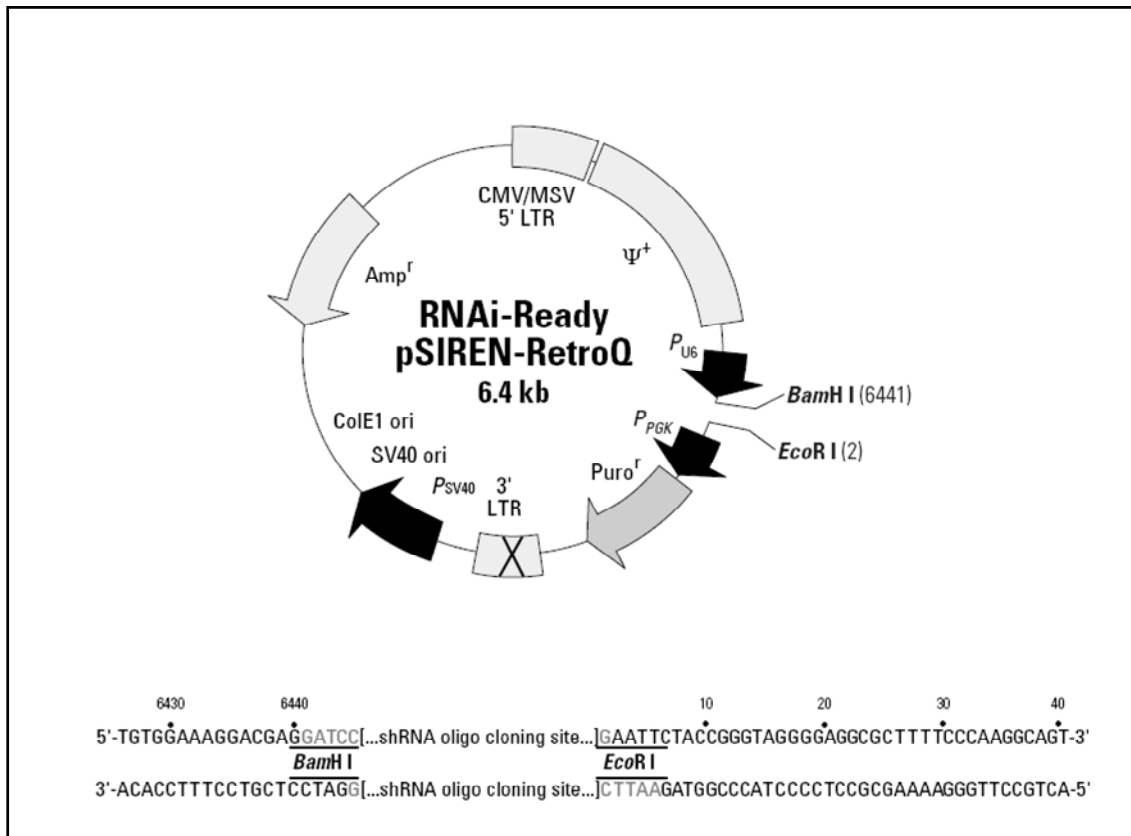
Figure 7-3: RNAi-Ready pSIREN-RetroQ vector. A self-inactivating retroviral expression vector designed to express a small interfering double stranded RNA (shRNA) using the human U6 promoter.

7.2 Antibodies

antibody	species	antigen, source	final dilution
gp-anti-Col XVI	Guinea pig, polyclonal antiserum, protein A purified	Full length recombinant collagen XVI	IB: 1:1000 IF (colon sections): 1:10 +TSA enhancer kit IF (cells): 1:75
Strep II	Mouse, monoclonal	StrepII-Tag (GenScript, USA)	IB: 0.1 μg/ml
AGF1	Mouse, monoclonal	α1-integrin (kind gift from Johannes A. Eble)	FC: 6.3 μg/μl
MAB1973Z	Mouse, monoclonal	α1-integrin (Millipore,	IF:1:150

		Germany)	
JA221	Mouse, monoclonal	α2-integrin (kind gift from Johannes A. Eble)	FC: 2.6 µg/µl
CD49bAK-7	Mouse, monoclonal	α2-integrin (Bio-Legend, USA)	FC: 1:25
mAB13	Rat, polyclonal	β1-integrin (kind gift from Johannes A. Eble)	FC: 1:1000
alpha 10	Mouse, monoclonal	α10-integrin (kind gift of Evi Lundgren-Ackerlund)	FC: 0.57 µg/µl
alpha 11	Rabbit, polyclonal	α11-integrin (kind gift of Evi Lundgren-Ackerlund)	FC: 1:500
ms-anti-Vinc	Mouse, monoclonal	Human vinculin (Sigma-Aldrich, USA)	IF: 1:500
rbt-anti-Vinc	Rabbit, polyclonal	Human vinculin (Sigma-Aldrich, USA)	IF: 1:400
rbt-anti-PPIA	Rabbit, polyclonal	Human peptidyl-prolyl-cis-trans isomerise A (Acris, USA)	IB: 1:500
rbt-anti-MIF	Rabbit, polyclonal	Human macrophage migration inhibitory factor (Santa Cruz, USA)	IB: 1:200
ms-anti-SMA	Mouse, monoclonal	Human smooth muscle actin clone 1A4 (DAKO, Denmark)	IF:1:25
ms-anti-FN	Mouse, monoclonal	Human fibronectin IgG1 (Acris, USA)	IF:1:20

Table 7-1: List of primary antibodies. gp: guinea pig; rbt: rabbit; ms: mouse; IB = Immunoblot; IF = Immunofluorescence; FC = flow cytometry

antibody	final dilution
Goat-anti-gp Alexa Fluor® 555, Molecular Probes, USA	5 µg/ml
Goat-anti-ms Alexa Fluor® 488, Molecular Probes, USA	5 µg/ml
Goat-anti-ms Alexa Fluor® 568, Molecular Probes, USA	5 µg/ml
Goat-anti-gp biotinylated antibody, Sigma-Aldrich, USA	1:800
Phalloidin-Alexa Fluor® 568, Sigma-Aldrich, USA	1:40
DAPI Nucleic Acid Stain, Molecular Probes, USA	300 nM

Table 7-2: List of secondary antibodies used for immunofluorescence.

gp: guinea pig; rbt: rabbit; ms: mouse

antibody	final dilution
Goat-anti-gp peroxidase conjugated (Sigma-Aldrich, USA)	1:30,000
Goat-anti-ms peroxidase conjugated (Pierce, USA)	1:30,000
Goat-anti-rbt peroxidase conjugated (Pierce, USA)	1:30,000

Table 7-3: List of secondary antibodies used for immunoblotting.

gp: guinea pig; rbt: rabbit; ms: mouse

8. Statutory declaration

I declare that this thesis was carried out all by myself and that no other means or resources were utilized than indicated in this work.

Regensburg,

Sabine Ratzinger

9. Curriculum vitae

Personal Information

Family Name	Ratzinger
First Name	Sabine
Birth Date	June 25, 1978
Nationality	German
Marital Status	Single

Educational Background

2004-	Dr.rer.nat.; Pharmacology and Toxicology, University of Regensburg, Germany
1998-2003	Diploma in Biotechnology at the University of Applied Science in Weihenstephan / Freising
	Diploma Thesis:
	"Investigations on the growth and stimulation conditions of cells as a preparation for the establishment of an endothelial cell bioreactor for experimental sepsis"
	Centre for Biomedical engineering, Danube-University Krems, Austria
1994-1998	Higher secondary school; Gymnasium Untergriesbach
1990-1994	Secondary school; Johann-Riederer-Realschule Hauzenberg
1984-1990	Primary school; Grund- und Hauptschule Untergriesbach

Work Experience

07/2007-	Research Associate and Head of the Laboratory; work group "Tissue engineering", Department of Otorhinolaryngology, University Hospital of Regensburg, Germany

01/2004-06/2007	Doctoral research at the Department of Pharmacology and Toxicology; University of Regensburg, Germany, lead by Prof. Dr. Roland Seifert under the direct supervision of PD Dr. Susanne Grässel, Department of Orthopaedic Surgery, University Hospital Regensburg, Germany
08/2003-11/2003	Technical assistant in R&D department of Applied Science at Roche Diagnostics, Penzberg, Germany
09/2002-06/2003	Diploma Thesis at the Centre for Biomedical engineering, Danube-University Krems, Austria
08/2001-12/2001	Internship at the Centre Centre for Biomedical engineering, Danube-University Krems, Austria
03/2000-07/2000	Internship Milan GmbH & Co KG, Institute for Analytical microbiology and quality control in Passau, Germany

Academic Awards and Honours

July 2008	GlaxoSmithKline travel fellowship
July 2007	GlaxoSmithKline travel fellowship
July 2006	Travel fellowship from "Friends of the University of Regensburg" GlaxoSmithKline travel fellowship
March 2006	Poster prize at the Annual Meeting of the German Connective Tissue Society, Tübingen, Germany, "Collagen XVI in Crohn's disease: TGFß-induced expression in intestinal myofibroblasts and cell adhesion properties"

Grants

2009	Grant from Deutsche Krebshilfe "Role of collagen XVI in the oral squamous cell carcinoma" in collaboration with the Department of Maxillofacial surgery and the Department of Orthopaedic surgery

2007   Scholarship of the Max-Buchner-Forschungsstiftung; #2734: „In vitro gene silencing of collagens in neuronal cells and glia cells"

Publications

Ratzinger S, Eble J A, Pasoldt A, Opolka A, Rogler G, Grifka J, Grässel S
Collagen XVI induces formation of focal contacts on intestinal myofibroblasts isolated from the normal and inflamed intestinal tract.
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Opolka A, Ratzinger S, Schubert T, Spiegel HU, Grifka J, Bruckner P, Probst A, Grässel S.

Collagen IX is indispensable for timely maturation of cartilage during fracture repair in mice.

Matrix Biol. 2007 Mar;26(2):85-95. Epub 2006 Oct 3

Abstracts presented at conferences

Posters

"Establishement of an in vitro larynx model for vocal fold augmentation"
Annual Meeting of the German Society of Otorhinolaryngology and Head and Neck Surgery, Rostock, Germany, May 20-24th 2009

"Collagen XVI – a new marker in oral squamous cell carcinoma?"
Annual Meeting of the German Society of Otorhinolaryngology and Head and Neck Surgery, Rostock, Germany, May 20-24th 2009

"Collagen XVI induces number and length of focal contacts formed by intestinal myofibroblasts isolated from the normal and inflamed intestinal tract"
Annual Meeting of the German Connective Tissue Society, Reims, France, June 3-15th 2009

"Intestinal myofibroblasts exhibit altered proliferation and adhesion properties during inflammatory progression in Crohn's disease"
Annual Meeting of the German Connective Tissue Society, Freiburg, Germany, April 3-5th 2008
XXIst FECTS meeting, Marseille, France, July 9-13th 2008

"Functional analysis of retroviral mediated knockdown of collagen XVI in NIH3T3 cells"
Annual Meeting of the German Connective Tissue Society, Freiburg, Germany, April 3-5th 2008
XXIst FECTS meeting, Marseille, France, July 9-13th 2008

"Collagen XVI in Crohn's Disease"
Collagen Gordon Research Conference in New London/USA, July 22nd-27th 2007

"Autologous platelet-rich plasma (PRP) for cartilage tissue engineering"
Annual Meeting of the German Connective Tissue Society, Münster, Germany, March 9-10th 2007

"Collagen XVI in Crohn's disease: adhesion properties of intestinal myofibroblasts"
Annual Meeting of the German Connective Tissue Society, Münster, Germany, March 9-10th 2007

"TGFß-3 induced expression of collagen XVI and cell adhesion properties of intestinal myofibroblasts under physiological and pathological conditions"
XXth FECTS and ISMB meeting, Oulu, Finland, July 1-5, 2006

"Collagen XVI in Crohn's disease: TGFß-induced expression in intestinal myofibroblasts and cell adhesion properties"
Annual Meeting of the German Connective Tissue Society, Tübingen, March 9-11th 2006

"Expression and allocation of collagen XVI in inflammatory bowel diseases"
First Joint Meeting of the French and German Connective Tissue Societies
Cologne, March 10-12th 2005

Oral presentations

"Collagen XVI – a new marker in oral squamous cell carcinoma?"
Annual Meeting of the German Society of Otorhinolaryngology and Head and Neck Surgery, Rostock, Germany, May 20-24th 2009

"Intestinal myofibroblasts exhibit altered proliferation and adhesion properties during inflammatory progression in Crohn's disease"

Annual Meeting of the German Connective Tissue Society, Freiburg, Germany, April 3-5th 2008

"Intestinal myofibroblasts exhibit altered proliferation and adhesion properties during inflammatory progression in Crohn's disease"
XXIst FECTS meeting, Marseille, France, July 9-13th 2008

"Collagen XVI – of mice and men"
Invited lecture, SFB 492 Extrazelluläre Matrix: Biogenese, Assemblierung und zelluläre Wechselwirkungen, Institut für Physiol. Chemie u. Pathobiochemie, Münster, November 20th 2006

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