TECHNISCHE UNIVERSITÄT MÜNCHEN
DEPARTMENT CHEMIE
LEHRSTUHL FÜR BIOCHEMIE

Epithelial-stromal interaction in pancreatic cancer: The role of collagen type V

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Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. Aymelt Itzen

Prüfer der Dissertation:

1. Univ.-Prof. Dr. Michael Groll
2. Univ.-Prof. Dr. Irene Esposito

Die Dissertation wurde am 24.09.2014 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 05.02.2015 angenommen.

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Abstract

Pancreatic ductal adenocarcinoma is a very aggresive disease with a poor prognosis. It is characterized by a dense desmoplastic stromal reaction, which is mainly produced by pancreatic stellate cells (PSC) and consists of extracellular matrix (ECM) proteins, including collagens. Collagen type V (Col V) is part of the desmoplastic stroma in pancreatic ductal adenocarcinoma and has already been described in other tumors types. However, the exact role of Col V in the epithelial-stromal interactions in pancreatic ductal adenocarcinoma is still unknown.

In this work, the function of Col V in the stroma of pancreatic ductal adenocarcinoma and its role in the interaction between cancer cells and PSC was investigated.

First, Col V expression was tested during tumor progression by immunohistochemistry in a large cohort of pancreatic ductal adenocarcinoma (PDAC) patients and in precursor lesions. Immunofluorescence and immunoblotting were used to investigate the expression of Col V in PSC and pancreatic cancer cells (PCC). In order to gain a more detailed understanding of the importance of endogenous Col V expression, fibroblasts bearing a Col V mutation (obtained from patients affected by the classical Ehlers-Danlos syndrome) were compared to PSC by migration and adhesion assays. The Col V-dependent downstream signaling pathway was then analyzed using immunoblotting, immunohistochemistry and immunocytochemistry. *In vitro* assays were performed to evaluate the effects of exogenous Col V on adhesion, proliferation and migration of cancer cells. Epithelial-stromal interactions were investigated by assessing angiogenesis as well as tumor cell invasion and proliferation after knock-down of Col V in PSC. A stable knock-down was used to test the effect of Col V on angiogenesis and proliferation of primary tumors and metastasis formation *in vivo* after orthotopic implantation of PSC and cancer cells in the tail of the pancreas of immunodeficient mice.

Col V was found to be up-regulated during tumor progression and PSC were confirmed as the main source of Col V in tumor tissues. Assays with fibroblasts bearing a Col V mutation revealed the important role of an intact endogenous Col V expression on the behavior of fibroblasts. Col V-mediated activation of the β_1-integrin signaling pathway with its downstream targets focal adhesion kinase and paxillin was found to promote adhesion, proliferation and migration of PCC *in vitro*. These effects could be partially blocked by a Src inhibitor and a blocking antibody against β_1-integrin. In addition, Col V was found to affect the tube formation of human umbilical vein endothelial cells (HUVEC). *In vivo*, the knock-down of Col V led to a decreased number of microvessels and metastatic foci in the liver.

Taken together, these results indicate an important role of Col V in PSC-mediated angiogenesis as well as promotion of the malignant phenotype of pancreatic ductal adenocarcinoma *via* activation of the β_1-integrin signaling pathway.

Zusammenfassung

Beim duktalen Adenokarzinom des Pankreas (*pancreatic ductal adenocarcinoma*, PDAC), handelt es sich um eine sehr aggressive Krebsart mit äußerst schlechter Prognose. PDAC ist charakterisiert durch ein desmoplastisches Stroma, reich an Kollagenen und anderen extrazellulären Matrixproteinen, welche hauptsächlich durch pankreatische Stellatumzellen (PSC) sezerniert werden. Kollagen Typ V (*collagen type V*, Col V) wurde bereits in verschiedenen Tumorentitäten als Komponente des desmoplastischen Stromas beschrieben. Die genaue Rolle von Col V in der epithelialen-stromalen Interaktion im Pankreaskarzinom ist jedoch bislang noch unbekannt. In dieser Arbeit sollte daher die Funktion von Col V im Stroma des PDAC genauer untersucht und dessen Rolle in der Interaktion zwischen Karzinomzellen und PSC ermittelt werden.

Zunächst wurde die Expression von Col V während der Tumorprogression von Vorläuferläsionen bis hin zum invasiven PDAC in einer großen Kohorte von PDAC Patienten immunhistochemisch analysiert. Die Expression von Col V in PSC und pankreatischen Karzinomzellen wurde mittels Immunfluoreszenzfärbungen und Immunblotting untersucht. Darüber hinaus wurden Fibroblasten mit einer Col V Mutation (isoliert aus Patienten mit Ehlers-Danlos Syndrom), mit PSC verglichen um ein besseres Verständnis über die Bedeutung von intaktem endogenem Col V zu erhalten. Der Signalweg *downstream* von Col V wurde anschließend mittels Immunblotting, Immunhistochemie und Immunzytochemie ermittelt. Verschiedene *in vitro* Assays wurden verwendet um den Effekt von exogenem Col V auf die Adhäsion, Proliferation sowie Migration verschiedener Pankreaskarzinomzellen zu testen. Der Einfluss auf die epitheliale-stromale Interaktion wurde mittels eines Col V *knock-downs* in PSCs anhand der Effekte auf Angiogenese sowie Tumorzell-Invasion und Proliferation untersucht. Mit Hilfe eines stabilen *knock-downs* von Col V in PSCs wurden die Auswirkungen auf die Angiogenese, Tumorwachstum und Metastasierung *in vivo* in einem orthotopen Mausmodell analysiert. Hierbei wurden Karzinomzellen zusammen mit PSC in den Pankreasschwanz von immundefizienten Mäusen injiziert.

Es konnte gezeigt werden, dass die Col V Expression im Verlauf der Tumorprogression anstieg. PSC wurden als Hauptquelle von Col V in PDAC identifiziert. Die wichtige Rolle des intakten endogenen Col V auf das Verhalten der Fibroblasten konnte mit dem mutierten Fibroblasten bestätigt werden. Die Col V-vermittelte Aktivierung des β_1-Integrin-Signalweges und der *downstream* Effektoren FAK und PAX führte zu einer erhöhten Adhäsion, Proliferation und Migration von Pankreaskarzinomzellen *in vitro*. Diese Effekte waren teilweise durch die Verwendung eines Src-Inhibitors sowie eines β_1-inhibitorischen Antikörpers blockierbar. Darüber hinaus unterstütze Col V die Tube Formation von humanen umbilikalen venösen Endothelzellen (*human umbilical vein endothelial cell*, HUVEC) *in vitro*.

Ein knock-down von Col V in PSCs *in vivo* führte zu einer reduzierten tumoralen Mikrogefäßdichte sowie zu einer verringerten Anzahl von Lebermetastasen.

Zusammen genommen weisen diese Ergebnisse auf eine wichtige Rolle von Col V in der PSC-vermittelten Angiogenese sowie in der Förderung des malignen Phänotyps von PDAC durch die Aktivierung des β_1-Integrin-Signalweges hin.

1 Introduction

1.1 Pancreatic cancer

Many different subtypes of pancreatic cancer are described, but pancreatic ductal adenocarcinoma (PDAC) is the most common and deadliest form (Hansel, Kern et al. 2003), representing more than 90 % of the malignant tumors in the pancreas (Bosman F.T. 2010). PDAC is a very lethal disease with a poor prognosis. In the United States, approximately 46 420 patients will be diagnosed with PDAC in 2014 and nearly an equal number (39 590) will die from this disease. The five-year survival rate is about 7 %, making this cancer the fourth leading cause of cancer death in the United States, where the incidence and mortality rates in men and women are equal (Siegel, Ma et al. 2014). Reasons for this dismal prognosis are the lack of early detection, the anatomic site of the pancreas with the retroperitoneal infiltration occurring early on during cancer development, nerve invasion, early distant tumor growth and therapy resistance. At diagnosis, local infiltration and metastasis are usually already present. To date, complete surgical resection represents the only chance for cure (Beger, Rau et al. 2003).

1.1.1 Diagnosis and therapy

PDAC is usually diagnosed at late stages because it often develops without early specific symptoms with the exception of tumors obstructing the bile duct, thus causing painless jaundice already in early stages (Herold 2013). Other symptoms include abdominal or back pain, weight loss, poor appetite, digestive problems and recent-onset diabetes. Treatment options for PDAC are surgery, chemotherapy and radiotherapy, depending on the stage of the disease. To date, the only curative treatment option for this cancer entity is surgery. Most patients are diagnosed in later stages, where only palliative approaches are feasible. Clinical trials could show that adjuvant therapy with gemcitabine prolongs survival after surgery (Burris, Moore et al. 1997). Further, the combination therapy of gemcitabine and erlotinib demonstrates a small improvement, compared to gemcitabine alone, in advanced PDAC with a median survival of 6.24 months versus 5.91 months (Moore, Goldstein et al. 2007). To date, gemcitabine and erlotinib are registered for treatment of advanced PDAC (Heinemann, Haas et al. 2012). However, FOLFIRINOX a combination of 5-FU/folfinic acid, irinotecan and oxaliplatin compared to gemcitabine alone showed a survival of 6.4 months versus 3.3 months in randomized phase III trial (Conroy, Desseigne et al. 2011).

1.1.2 Risk factors/Epidemiology

Until now, various risk factors for PDAC have already been identified, which include advanced age, race, alcohol abuse, smoking, diet, obesity, diabetes, chronic pancreatitis (CP), hereditary and

infectious diseases. Commonly, PDAC is a disease that is rare before the age of 40, the median age of onset is 71 years and the rates of PDAC are increased in the black population (Koorstra, Hustinx et al. 2008, Yadav and Lowenfels 2013). A major risk factor is CP, here the relative risk is 13.3 (Raimondi, Lowenfels et al. 2010). In particular, hereditary pancreatitis plays an important role. The cumulative risk of these patients until the age of 70 is 40 % (Lowenfels, Maisonneuve et al. 1997, Weiss 2014). Other hereditary syndromes are also described as risk inducing factors (see table 1). Smokers have a 2.2 higher risk of PDAC compared to never smokers and the risk increases in proportion to smoking (Bosetti, Lucenteforte et al. 2012). In smokers PDAC occurs approximately 10 years earlier than in nonsmokers (Boyle, Maisonneuve et al. 1996). For high-fat diet, a correlation between the intake of saturated fat and the incidence of PDAC was found (Farrow and Davis 1990, Lyon, Slattery et al. 1993). Diabetes of type 2 is another risk factor for the development of PDAC (Brand and Lynch 2004), a possible explanation being the mitogenic effects of high concentrations of insulin (Draznin 2010); further obese people have a relative risk of 1.19 to develop pancreatic cancer compared to people with normal body weight (Berrington de Gonzalez, Sweetland et al. 2003).

Table 1: Hereditary syndromes of PDAC and the reported mutated genes

Syndrome	Gene
Familial atypical multiple mole melanoma (FAMMM)	*CDKN2*
Peutz-Jeghers syndrome (PJS)	*STK11*
Hereditary pancreatitis (HP)	*PRSS1, SPINK1*
Hereditary non-polyposis colorectal carcinoma (HNPCC)	*MLH1, MSH2* and others
Familial breast and ovarian cancer (FOBC)	*BRCA1, BRCA2*
Cystic fibrosis (CF)	*CFTR*
Ataxua-thelangiectasia (AT)	*ATM*
Familial adenomatous polyposis (FAP)	*APC*
Familial pancreatic cancer (FPC)	*BRCA2*

1.1.3 Precursor lesions

To date, PanIN (pancreatic intraepithelial neoplasia), IPMN (intraductal papillary mucinous neoplasms) and MCN (mucinous cystic neoplasms) are described as the three most important precursor lesions (Hruban, Maitra et al. 2007). Atypical flat lesions (AFL) have been recently proposed as additional precursor lesions (Aichler, Seiler et al. 2012).

PanIN

The most common and best characterized lesions are the PanINs (pancreatic intraepithelial neoplasia), which are subdivided in PanIN-1-3 according to the degree of cytological and architectural atypia. PanIN-1A are flat epithelial lesions with basally located nuclei and abundant cytoplasmic mucin. PanIN-

1B are similar to PanIN-1A, but have a micropapillary/papillary architecture and slightly pseudostratified nuclei. PanIN-2 lesions have a mucinous epithelium and are mostly papillary lesions. The nuclei show some abnormalities like loss of polarity, crowding and enlargement, pseudostratification and hyperchromatism. PanIN-3 are papillary or micropapillary and may display small clusters of epithelial cells budding off into the lumen. They are characterized by loss of polarity, nuclear enlargement, by hyperchromatism and increased numbers of mitotic figures, which can be abnormal. PanIN-3 show cytonuclear alterations similar to those observed in PDAC, but invasion to the basement membrane is not seen at this stage.

This progression in the degree of dysplasia is accompanied by the accumulation of genetic alterations as shown in figure 1 (Maitra, Adsay et al. 2003). Frequencies of the mutations found in PDAC are summarized in table 2.

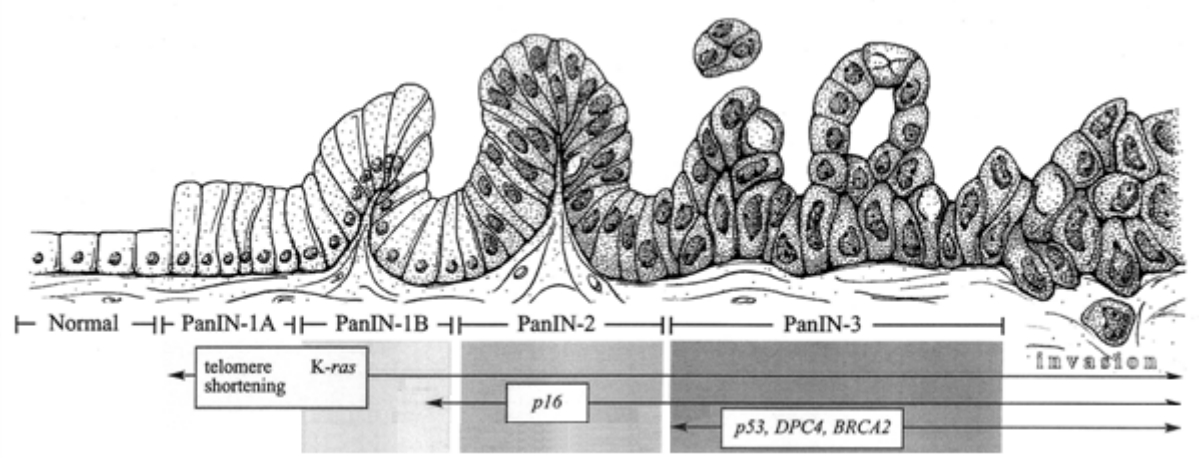

Figure 1: Progression model for PDAC from normal epithelium through PanINs. In the early stages *KRAS* mutations are present, in the intermediate stage an additional mutation of *CDKN2A* occurs. The mutations of *TP53*, *SMAD4* and *BRCA2* take place in late stages (Maitra, Adsay et al. 2003).

Table 2: Frequency of mutations in PDAC (Ottenhof, de Wilde et al. 2011)

Gene	Frequency of mutations in PDAC
KRAS	> 90 %
CDKN2A	95 %
TP53	75 %
SMAD4	55 %

IPMN

IPMN are intraductal papillary neoplasms with atypical epithelium that produces mucin. They are at least 1 cm in size and involve the main pancreatic duct or one of its branches (Bosman 2001). IPMN may display low grade, moderate and high-grade dysplasia (Hruban, Maitra et al. 2007) and classified

into 4 histological subtypes: gastric, intestinal, pancreatobiliary and oncocytic. The 5-year survival rate is dependent on the histological subtype, which ranges from 52 % for the pancreatobiliary to 94 % for the gastric subtype (Schlitter and Esposito 2012).

MCN

MCN are cyst-forming epithelial neoplasms that produce mucin and have an ovarian-type stroma. Their size ranges between 1 to 3 cm and the cysts contain mucin or hemorrhagic fluid. One third of the cases are associated with invasive carcinoma (Wilentz, Albores-Saavedra et al. 2000). The 5-year survival rate is almost 100 % for resected patients with non-invasive carcinoma. For patients with resected invasive carcinoma the 5-year survival-rate is 50 to 60 % (Le Borgne, de Calan et al. 1999).

AFL

A further possible alternative pathway of pancreatic carcinogenesis was discovered recently. Initially, AFL were found in genetically modified mouse models for PDAC harboring a pancreas specific *Kras* mutation. Here, the AFL are formed in tubular complexes (TC) originating in areas of acinar-ductal metaplasia (ADM). Their relevance in human patients was confirmed in some cases of familial pancreatic cancer. Compared to the low grade PanIN lesions, AFL have an increased proliferation rate and nuclear p53 expression. AFL with these characteristics could not be found in sporadic PDAC tissues, thus leading to the possibility that AFL might present new and additional precursor lesions of PDAC. Morphologically, characteristic of AFL are tubular structures with flattened epithelium and enlarged and hyperchromatic nuclei in areas of ADM. AFL are surrounded by a peculiar stroma with characteristic overexpression of ECM proteins (Aichler, Seiler et al. 2012).

1.1.4 Genetics of PDAC

Along with the changes in morphology, the precursor lesions acquire genetic alterations that accelerate the progression to invasive cancer. In the progression of PDAC it is known that each step is likely to be associated with specific genetic alterations. Some of these mutations are thought to influence the progression of the precursor lesions more than the cancer initiation, because the typical age of PDAC incidence is high (Hezel, Kimmelman et al. 2006).

KRAS mutations are the first genetic alterations that can be detected in early lesions. To date different *KRAS* mutations are found, these include mutations at codon 12, 13 and 61. For example, the sporadically activating point mutation at codon 12 leads to a substitution of glycine with aspartate, valine or arginine. This mutation at codon 12 can already be found in 92.0 % of PanIN-1A lesions (Kanda, Matthaei et al. 2012). Even if this mutation is already present at early stages with a high incidence, it is known that healthy people also have a high incidence in *KRAS* mutations; however this is much higher than the rates of cancer development. Pointing this out, mutations in the *KRAS* gene locus alone seems not to be sufficient to transform a cell. Further, mutated KRAS is not feasible as a

biomarker, as the oncogenic mutations are commonly acquired with age (Parsons and Meng 2009). Nevertheless, as *KRAS* has to be activated for the downstream signaling, it could be used as a target for therapeutic agents (di Magliano and Logsdon 2013). Hingorani et al. used the knowledge of *KRAS* mutation to engineer a mouse model that develops murine PanINs (Hingorani, Petricoin et al. 2003), but these mice alone rarely develop cancer, which is consistent to the situation in humans, as they also need an accumulation of genetic alterations to develop PDAC (Jones, Zhang et al. 2008). A further interesting finding is that *KRAS* also influences the microenvironment. Collins et al. showed that recruited fibroblasts stop expressing markers of activation, exit the cell cycle and eliminate from the pancreas when *KRAS* is inactivated in an inducible mouse model (Collins, Bednar et al. 2012).

ERBB2 overexpression was first described by Hall et al. (Hall, Hughes et al. 1990). Later, it was shown that it is prominent from early stage precursor lesions on and appears to correlate with the grade of dysplasia (Day, Digiuseppe et al. 1996).

Different genetic alterations are known for *CDKN2A*, these include homozygous deletion, somatic mutations and hypermethylation (Ohtsubo, Watanabe et al. 2003). The relative risk for the development of PDAC is almost 50 times greater with a *CDKN2A* mutation (de Snoo, Bishop et al. 2008). The germline mutation of *BRCA2* is associated with familial breast and ovarian cancer and a higher risk of PDAC. *BRCA2* mutations are found in 17 % of familial PDACs. The loss of the second allele is a late event in the tumor progression. Mutations of the tumor suppressor gene *TP53* occur in later phases of tumor progression (PanIN-3 and PDAC), mostly through missense mutations. Morton et al. showed that *TP53* is more often mutated than deleted. This proves that p53 can act as an oncogene and may give advantages in tumor cell growth. The mutation has an incidence of 50-75 % of the tumors (Morton, Timpson et al. 2010).

SMAD4 is a tumor suppressor that regulates the TGF-β signaling pathway and inhibits the growth of epithelial cells (Hahn, Schutte et al. 1996, Massague, Blain et al. 2000). It is lost in about 55 % of PDAC (Tascilar, Skinner et al. 2001).

1.1.5 Microenvironment

The hallmark characteristic of PDAC is a dense stromal reaction surrounding and sustaining the tumor, making the treatment very challenging. Due to the desmoplastic stroma, the cancer is poorly perfused and vascularized and therefore may act as a barrier for drug delivery and provide subsequent resistance to chemotherapy. Stroma depletion has been suggested as a possible strategy for successful chemotherapy (Olive, Jacobetz et al. 2009, Neesse, Michl et al. 2011). Recently, a new drug, nab-paclitaxel, has been used to deplete the stroma (Hawkins, Soon-Shiong et al. 2008). Nab-paclitaxel is an albumin-bound drug formulation; it is assumed that the uptake into the cancer tissue can be facilitated through binding of albumin to SPARC (secreted protein acidic and rich in cysteine)

(Gradishar, Tjulandin et al. 2005), which is an ECM protein expressed both in the stromal and in the cancer cells in PDAC (Infante, Matsubayashi et al. 2007). However, recent data suggest that depletion of the stromal component promotes tumor progression, indicating that the stromal response might actually act as a barrier against neoplastic growth. In the study of Rhim et al. the depletion of Shh led to reduced stromal desmoplasia, followed by accelerated tumor growth, increased systemic morbidity and metastasis formation (Rhim, Oberstein et al. 2014). In another recent study depletion of $aSMA^{+}$ myofibroblasts led to undifferentiated tumors with enhanced hypoxia and more aggressive clinical behavior (Ozdemir, Pentcheva-Hoang et al. 2014).

The desmoplastic stroma of PDAC shows a high heterogeneity and consists of cellular (fibroblasts, PSC and immune cells) as well as extracellular components, such as ECM proteins, cytokines and growth factors. During tumor progression, the composition of the tumor microenvironment is altered. This is mainly due to an increased production of ECM proteins (collagens, fibronectins, proteoglycans, hyaluronic acid, active enzymes and proteinases) by PSC. Through this desmoplastic reaction an increased number of blood and lymphatic vessels arises, which further sustains tumor growth (Feig, Gopinathan et al. 2012).

With the *Kras* mouse model (*PDX-1-Cre;LSL-KRAS^{G12D}* and *P48$^{+/Cre}$;LSL-KRAS^{G12D}*) established by Hingorani et al. a promising tool for the investigation of PDAC in the murine system was found, which recapitulates the morphology of the human disease. Here, a pancreas specific *Kras* mutation (*KRAS^{G12D}*) is achieved using the Lox-Stop-Lox system. A Cre recombinase that is expressed from a pancreatic-specific promoter, *PDX-1* or *P48*, removes the Stop cassette and therefore leads to the translation of the mutation only in pancreatic tissue. Clark and coworkers could show in this model that the progression of the disease was accompanied by a progressive infiltration of leukocytes making up about 50 % of the tumor mass (Clark, Hingorani et al. 2007), suggesting that additionally to the stromal reaction, inflammation plays a critical role in PDAC. In the tumor microenvironment cells of the innate and the adaptive immune system can be found with sometimes opposite functions (de Visser, Eichten et al. 2006), e.g. macrophages, mast cells, neutrophil granulocytes, dendritic cells, and B- and T-lymphocytes (Esposito, Kleeff et al. 2002, Esposito, Menicagli et al. 2004, Li, Kleeff et al. 2004, Michalski, Gorbachevski et al. 2007). Recruitment of cells of the innate immune system can be induced by the cancer cells themselves, e.g. through TGF-β1 or VEGF. T-cells can recognize several cancer-associated antigens that are expressed by PDAC (Kubuschok, Xie et al. 2004, Schmitz-Winnenthal, Volk et al. 2005). Inflammatory cells, mainly macrophages may contribute to tumor growth, invasion and metastasis. They are for example the source of growth factors that stimulate proliferation (Pollard 2004). Mast cells and macrophages further were shown to increase the microvessel density, which in turn affected patients' prognosis (Esposito, Menicagli et al. 2004).

1.1.6 Pancreatic stellate cells

Using an electron microscopy, PSC have been described 30 years ago for the first time in the rodent and human pancreas (Watari, Hotta et al. 1982). Not before 1998, PSC were successfully isolated independently by two groups (Apte, Haber et al. 1998, Bachem, Schneider et al. 1998). The analogue of this cell type had already been found in the liver 130 years ago by Karl Wilhelm von Kupffer and had been described again 75 years later by Ito (Ito 1951) as lipid-containing cells. Stellate cells were also found in other organs, for example in the lung and kidney (Wake 1980). In the healthy pancreas, PSC compose 4-7 % of the tissue and are localized around the acini (Apte, Haber et al. 1998). They can be distinguished from fibroblasts by several markers like desmin, vimentin, nestin and various neuroectodermal markers. Further, they show a well-developed rough endoplasmatic reticulum, collagen fibrils and lipid droplets (Apte, Pirola et al. 2012). PSC play an important role in building and sustaining the desmoplastic reaction in chronic pancreatitis and PDAC. In normal tissue, they appear in a quiescent state and show a polygonal shape with vitamin A storing lipid droplets. With exposure to UV light at 328 nm, the vitamin A droplets display a blue-green fluorescence. Upon injury, PSC activate and begin to secrete numerous ECM-proteins (Haber, Keogh et al. 1999), but also enzymes that can degrade the ECM proteins and their inhibitors (Phillips, McCarroll et al. 2003). In normal tissue, PSC are responsible for the balance in the tissue turnover and can switch from an activated state again back to the quiescent state. During injury, this balance is destroyed, leading to persistent activation and production of ECM proteins. To distinguish between activated and quiescent PSC, several markers have been proposed. Quiescent PSC are stained by desmin, nestin and glial fibrillary acidic protein (GFAP) antibodies (Apte, Haber et al. 1998). The most common marker for activated PSC is α-smooth muscle actin (αSMA). However, αSMA is more a marker for transdifferentiation into a myofibroblast-like phenotype than a generic marker for activated PSC (Krizhanovsky, Yon et al. 2008). All in all, there is still an urgent need for specific markers to distinguish fibroblasts, myofibroblasts, activated and quiescent PSC from each other. In 2011, a secretome study by Wehr et al. compared proteins secreted from quiescent and activated PSC. They investigated processes and pathways known to play a role in carcinogenesis and pancreatitis, such as wound healing, where they found 21 proteins upregulated in activated PSC compared to quiescent PSC. Furthermore, 33 proteins associated with inflammation were found to be upregulated. Other proteins whose secretion was increased in activated PSC were Col I, III, and IV and fibronectin as well as 40 proteins involved in invasion, angiogenesis, proliferation and apoptosis (Wehr, Furth et al. 2011).

Pancreatic cancer cells (PCC) are known to activate PSC. This activation can be induced by different factors that are released by PCC (Apte, Park et al. 2004), e.g. cytokines and growth factors. In addition, ethanol and its metabolites can induce PSC activation. Cytokines and growth factors known to activate PSC include:

- PDGF, TGF-β (Apte, Haber et al. 1999),
- Activin A (Ohnishi, Miyata et al. 2003),
- TGF-α, basic fibroblast growth factor (Bachem, Schneider et al. 1998),
- TNF-α (Schneider, Schmid-Kotsas et al. 2001)
- Interleukin-1 and interleukin-6 (Mews, Phillips et al. 2002).

Among them, TGF-β1 is responsible for the ECM synthesis (Schneider, Schmid-Kotsas et al. 2001), PDGF acts as a mitogen (Luttenberger, Schmid-Kotsas et al. 2000, Schneider, Schmid-Kotsas et al. 2001) and is responsible for the migratory capacity (Phillips, Wu et al. 2003). Producers of the mentioned cytokines are macrophages (Schmid-Kotsas, Gross et al. 1999), platelets (Luttenberger, Schmid-Kotsas et al. 2000) and acinar cells (Yu, Lim et al. 2002). Further, there is evidence that PSC can produce cytokines also in an autocrine way, that helps them to perpetuate their activated state (Kruse, Hildebrand et al. 2000).

Alcohol abuse is often the cause of chronic pancreatitis, leading to the activation of PSC and fibrosis. This is mediated through PSC activating proinflammatory cytokines (Apte and Wilson 2003); a direct effect of ethanol on PSC further contributes to their activation (Apte, Phillips et al. 2000) and causes αSMA expression and collagen synthesis. Figure 3 shows an overview of the activation processes from the quiescent PSC to their activated phenotype.

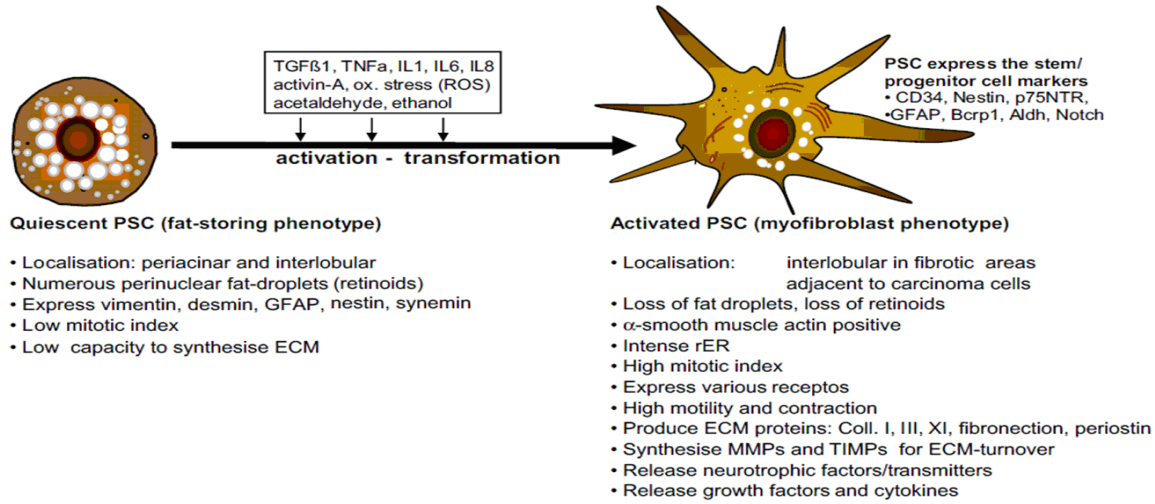

Figure 2: Scheme of the activation PSC (adapted from Erkan, Adler et al. 2012)

ECM components affect PSC through activation of signal transduction pathways. A better understanding of these pathways could help in developing new therapeutic strategies to inhibit PSC activation.

The mitogen activated protein kinase (MAPK) pathway is a focus of the researchers, as it is induced through several growth factors like Ras and Raf (Pearson, Robinson et al. 2001). ERK1/2 activation, which can be induced by PDGF, leads to a change in the phenotype of the PSC to a myofibroblast shape

(Jaster, Sparmann et al. 2002). Two other pathways are described to be involved in activation of PSC. One is the phosphoinositide 3-kinases pathway (PI3K), known to be involved in PSC migration but not proliferation. The other is the Rho-ROCK pathway that was shown to be involved in the *in vitro* activation of PSC through the regulation of the actin cytoskeleton (Masamune, Kikuta et al. 2003). To date, the studies about the activation of PSC are based on *in vitro* assays, which might not completely reflect the *in vivo* situation.

In addition to the influence that PCC have on PSC activation, they also can increase the proliferation, migration and ECM synthesis of PSC (Vonlaufen, Joshi et al. 2008). *Vice versa*, PSC are known to affect PCC in their migratory and proliferative behavior through the release of growth factors and cytokines. In addition PSC can inhibit apoptosis of cancer cells, giving them a pro-survival advantage. The effect that these two cell types have on each other was investigated in different *in vivo* studies, where PCC together with PSC were injected in orthotopic mouse models. In these experiments an increase in tumor size and higher incidence of metastasis was found (Hwang, Moore et al. 2008, Vonlaufen, Joshi et al. 2008, Xu, Vonlaufen et al. 2010). Moreover, it could be shown that the PSC were not only responsible for the tumor growth. In a study where male human PSC were injected together with female PCC in female mice, the PSC were also found at distant sites, leading to the point that they are either able to migrate together with the PCC in a cell cluster into the distant sites or that they travel separately and establish the microenvironment for the PCC (Xu, Vonlaufen et al. 2010).

1.2 Collagen type V

1.2.1 Collagen type V and its natural function

Collagens are structural ECM proteins and are an important component of the desmoplastic reaction in PDAC. Collagen type I and type III are two of the most extensively studied proteins produced by activated PSC. Also Col V has been recently discovered to be secreted by activated PSC (Wehr, Furth et al. 2011).

Col V is described as a minor fibril comprising only 2-5 % of the total collagens in most tissues, like cornea, tendon, dermis, bone and cartilage. In the cornea, it is more prominent and constitutes about 10-20 % of all collagens (Birk 2001, Segev, Heon et al. 2006). It belongs to the fibril forming collagens like collagen I, II, III and XI, containing an uninterrupted triple helical region. The collagens require several intracellular processes, like hydroxylation and glycosylation as well as extracellular ones like procollagen processing and cross-linking, to form fibrils (Birk 2001). These processes are common to all fibrillar collagens (Prockop and Kivirikko 1995). A schematic overview of Col V is shown in figure 3. One chain is composed of a thrombospondin N-terminal-like domain (TSPN), a nonhelical region (NHR), an interrupted collagenous region (ICR) and a fibrillar collagen C-terminal domain (ColF1). The triple-helical domain is located between the ICR and the NHR domain

Figure 3: Schematical view of the Col V chain. The protein consists of a thrombospondin N-terminal-like domain (TSPN), a nonhelical region (NHR), an interrupted collagenous region (ICR) and a fibrillar collagen C-terminal domain (ColF1).

Col V exists in different isoforms. The most common one is $\alpha1(V)_2\alpha2(V)$ which is found in the cornea (Birk, Fitch et al. 1988, Wenstrup, Florer et al. 2004). Further, a heterotrimer of all three chains $\alpha1(V)_1\alpha2(V)_1\alpha3(V)_1$ exists in the placenta and a $\alpha1(V)_3$ homotrimer is described only in Chinese hamster lung cells (Haralson, Mitchell et al. 1984).

The interaction between Col V and Col I is necessary for maintaining the tissue integrity and the formation of the Col I fibril, which differs in its diameter depending on the tissue. In comparison to the fibrillar collagens I-III, the Col V heterotrimer has a NH2-terminal globular sequence which protrudes beyond the fibrils and plays a role in fibrillogenesis, where it can regulate the Col I fibril diameter. The NH2 terminal domain is still accessible after fibril formation with Col I and could be labeled on isolated fibrils in a spiral distribution pattern (Birk, Fitch et al. 1988, Linsenmayer, Gibney et al. 1993). The process of the fibril formation is shown in figure 4.

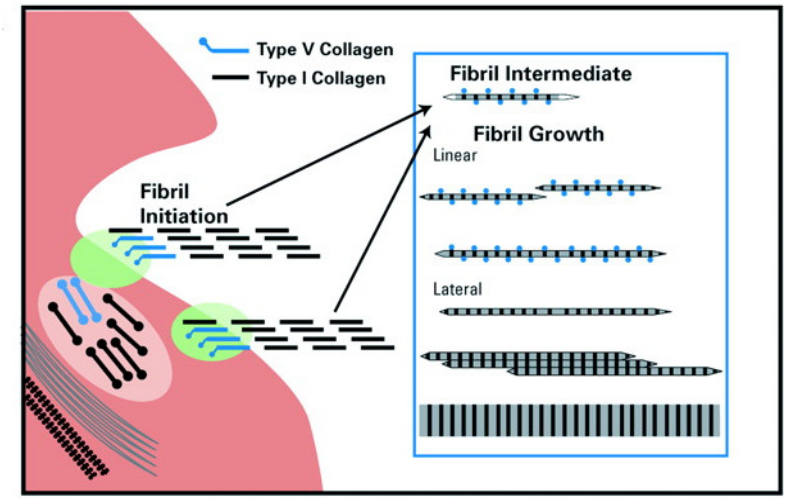

Figure 4: Fibril formation of Col I and Col V fibrils. The Col V fibrils are initiated at the surface of the connective tissue. The fibrils enlarge by lateral and end-to-end fusion (Wenstrup, Florer et al. 2004)

Mutations in the genes of the alpha 1 chain (*COL5A1*) and alpha 2 chain (*COL5A2*) of Col V are the main cause of the classical Ehlers-Danlos syndrome (Wenstrup, Florer et al. 2000). Most of them are nonsense or frameshift mutations causing premature termination and leading to a null-allele (Malfait,

Coucke et al. 2005, Mitchell, Schwarze et al. 2009). Further structural mutations, where the glycine in the triple helix is affected, were found. Splice mutations and homozygous missense mutations were described in the α1(V)-N-propeptide domain (Symoens, Malfait et al. 2009). Further, mutations in the C-propeptide are found (Malfait, Coucke et al. 2005). Glycine substitutions and in-frame exon skipping mutations have been reported in the triple helix-encoding domain (Bouma, Cabral et al. 2001). Most of these mutations cause haploinsufficiency and therefore the defective incorporation of Col V into the fibrils is leading to a decrease in fibril size (Wenstrup, Florer et al. 2004). A total Col V knock-out in mice results in lethality at embryonic day 10, probably because of cardiovascular failure. With a heterozygous knock-out, the mice develop features of the classical Ehlers-Danlos syndrome (Wenstrup, Florer et al. 2006), such as joint hypermobility, skin extensibility and tissue fragility (Beighton, De Paepe et al. 1998).

Numerous molecules have been described as receptors and/or binding partners of Col V. Ruggiero et al. described in 1996 that $\alpha_2\beta_1$-integrin is the main receptor and multiple sites of Col V can bind to it (Ruggiero, Comte et al. 1996, Zoppi, Gardella et al. 2004). Col V is known to bind to DNA, heparin sulfate, heparin (LeBaron, Hook et al. 1989), thrombospondin (Mumby, Raugi et al. 1984) and insulin (Yaoi, Hashimoto et al. 1991). Symoens et al. identified several additional binding partners of Col V by surface plasmon resonance and co-immunoprecipitation. These molecules were Col I and Col VI, tenascin-C, fibronectin, procollagen C-proteinase enhancer-1 (PCPE-1), tissue inhibitor of metalloproteinases 1 (TIMP-1), matrix metalloproteinase-2 (MMP-2) and TGF-β1 (Symoens, Renard et al. 2011).

1.2.2 Collagen type V signaling

As Col V is an ECM protein, the question arises what the dominant receptor for this molecule is. To date, very little information is available about Col V signaling. Murasawa et al. showed that β_1-integrin was localized in adhesion clusters at the migration edge of glomerular endothelial cells (Murasawa, Hayashi et al. 2008). Downstream targets of this pathway are the focal adhesion kinase (FAK) and paxillin (PAX). They are involved in the induction of cell migration and cell motility (Larsen, Tremblay et al. 2003). FAK can stimulate the turnover of the focal adhesions. First, FAK undergoes autophosphorylation at Tyr 397 (pFAK-Y397) through integrin stimulation. Then the kinase domain at Tyr 118 is phosphorylated (pPAX-Y118), which in turn activates Src. This stimulates the disassembly of α-actinin, induces cell detachment from the ECM thus promoting cell motility (Webb, Donais et al. 2004). These data show a potential mechanism for the outside-in signaling from Col V through β_1-integrin and the downstream activation of FAK and PAX, leading to a migratory phenotype of the cells when cultured on Col V (Murasawa, Hayashi et al. 2008).

1.2.3 Collagen type V and cancer

A collagen rich matrix has been described in some cancer types. For example, in skin tumors an increased content of Col V was found, whereas no difference in the level of Col I and Col III could be observed (Marian and Danner 1987). Expression of the alpha 2 chain of collagen type V (COL5A2) was seen to be associated with colorectal cancer. An increased level of COL5A2 in cancerous tissues together with the alpha 1 chain of collagen type XI (COL11A1) was seen in contrast to normal tissue, where no COL5A2 could be detected (Fischer, Stenling et al. 2001). Barsky et al. found an increased content of Col V in the desmoplasia of breast carcinoma. They also showed that Col V is not produced by the cancer cells but may be produced by myofibroblasts in the interstitium (Barsky, Rao et al. 1982). In breast cancer, further studies were performed to analyze the function of Col V in carcinogenesis. Luparello et al. discovered that Col V represents only 1 % of all collagens in normal breast tissue, but it is upregulated up to 10 % in invasive ductal carcinoma (Luparello, Rizzo et al. 1988). In further *in vitro* experiments using the cell line 8701-BC, they showed that Col V was a poorly-adhesive substrate for the cells and that the cells being able to attach showed a minor proliferation and motility (Luparello, Schillaci et al. 1990). Moreover, they observed that Col V is able to impair the survival of cancer cells through the promotion of a caspase-dependent apoptotic cell death (Luparello and Sirchia 2005). In a previous study, this group could show that the breast cancer cell line T47-D undergoes apoptosis when grown on Col V. However, the apoptosis-linked genes are different in the two analyzed breast cancer cell lines, implying heterogeneity in the transduction pathways induced by Col V. Other breast cancer cell lines MDA-MB231 and Hs578T, obtained from a carcinosarcoma and a non-ductal adenocarcinoma respectively, showed no apoptosis when cultured on Col V, indicating different responses depending on the tumor type and differentiation (Luparello, David et al. 2003). Further multiple and sometimes opposite responses have been described. For example Col V is an optimal substrate for hepatocytes (Takai, Hattori et al. 2001), Schwann cells (Chernousov, Stahl et al. 2001), normal smooth muscle cells (Grotendorst, Seppa et al. 1981) and different tumor cells (Ruggiero, Champliaud et al. 1994). On the other hand an anti-adhesive and anti-proliferative effect is shown on neurons (Chernousov, Stahl et al. 2001) and various epithelial and endothelial cells (Parekh, Wang et al. 1998, Underwood, Bean et al. 1998).

1.2.4 Aim of the study

The aim of this study was to investigate the role of Col V in the development and progression of PDAC, starting from its precursor lesions to invasive and metastasizing carcinoma. PSC, as the main tissue source of Col V *in vivo* were functionally characterized in comparison with different types of fibroblasts, including normal dermal fibroblasts and fibroblasts bearing a Col V mutation obtained by patients suffering from the Ehlers-Danlos syndrome. The role of Col V as growing substrate for PCC and the

downstream effectors of the Col V-mediated signaling pathway were characterized assessing their impact on functional properties of PCC *in vitro*. Angiogenesis, which is important for cancer growth and wound healing, was investigated to broaden the understanding of the role of Col V in this process. Finally, the effects of stable Col V knock-down in human PSC were tested on primary tumor growth and metastatic formation *in vivo*.

2 Material and methods

2.1 Material

2.1.1 Reagents

Product	*Company*
3,3'-Diaminobenzidine	Medac GmbH, Wedel (D)
5-Fluorouracil	Tocris Bioscience, Bristol (UK)
Acetic acid	Merck KGaA, Darmstadt (D)
Acrylamide/Bis Solution 40 %, 37.5:1	Bio-Rad Laboratories GmbH , Munich (D)
Adefofix fixing concentrate	Adefo-Chemie, Dietzenbach (D)
Adefofur developing concentrate	Adefo-Chemie, Dietzenbach (D)
Agarose LE for gel electrophoresis	Biozym Scientific GmbH, Hessisch Oldendorf (D)
Alexa Fluor 488 goat anti mouse IgG	Life Technologies GmbH, Darmstadt (D)
Alexa Fluor 546 goat anti rabbit IgG	Life Technologies GmbH, Darmstadt (D)
Alexa Fluor 647 goat anti rabbit IgG	Life Technologies GmbH, Darmstadt (D)
Ammonium persulfate	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Amphotericin B	Biochrom AG, Berlin (D)
Antibody diluent Dako REAL™	Dako Deutschland GmbH, Hamburg (D)
Basic fibroblast growth factor (bFGF)	BD Bioscience, Heidelberg (D)
BCA Protein Assay Kit	Thermo Fisher Scientific, Schwerte (D)
Biozym LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf (D)
Bovine Serum Albumin (BSA)	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Bromophenol blue	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Chloroform	Merck KGaA, Darmstadt (D)
Citric acid	Merck KGaA, Darmstadt (D)
Collagen from human placenta, Bornstein and Traub Type V (Sigma Type IX)	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Collagen type I from rat tail	Merck Millipore KGaA, Darmstadt (D)
Complete mini Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim, (D)
Deoxycholic acid	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Dimethylsulfoxid	Sigma-Aldrich Chemie GmbH, Steinheim (D)
DNA-Loading dye 6X	Thermo Fisher Scientific, Schwerte (D)
dNTP Set, 100 mM Solutions	Invitrogen™, Darmstadt (D)

Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (high glucose) 4.5 g/L	Gibco®, Darmstadt (D)
Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (low glucose) 1g/L	Gibco®, Darmstadt (D)
ECL mouse IgG, HRP-linked whole Ab (from sheep)	GE Healthcare, Munich (D)
ECL rabbit IgG, HRP-linked whole Ab (from sheep)	GE Healthcare, Munich (D)
Endothelial Growth Media	Promocell, Heidelberg (D)
Eosin	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Ethanol	Merck KGaA, Darmstadt (D)
Ethidium bromide	Amresco, Solon (USA)
Ethylene glycol tetraacetic acid	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Ethylenediaminetetraacetic acid	Sigma-Aldrich Chemie GmbH, Steinheim (D)
F12 Nutrient Mixture, L-Glutamin	Gibco®, Darmstadt (D)
Fetal Bovine Serum (FBS)	Gibco®, Darmstadt (D)
Formalin	Staub & Co. GmbH, Munich (D)
Gemcitabine	Tocris Bioscience, Bristol (UK)
GeneRuler™ 1 kb DNA Ladder	Thermo Fisher Scientific, Schwerte (D)
GeneRuler™ 100 bp DNA Ladder	Thermo Fisher Scientific, Schwerte (D)
Glycerin	Merck KGaA, Darmstadt (D)
Glycine	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Glycogen (30 mg/ml)	Peqlab Biotechnology GmbH, Erlangen (D)
Goat serum	Abcam plc, Cambridge, (UK)
Hemalm	AppliChem GmbH, Darmstadt (D)
HistoMark Biotin-Streptavidin Peroxidase Kit	KPL Inc., Gaithersburg (USA)
Hoechst 33342	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Hydrogen chloride	neoLab, Heidelberg (D)
Hydrogen peroxide 30 %	Merck KGaA, Darmstadt (D)
Isopropanol	Merck KGaA, Darmstadt (D)
Lipofectamine	Invitrogen™, Darmstadt (D)
Matrigel™	BD Bioscience, Heidelberg (D)
Methanol	Merck KgaA, Darmstadt (D)
Nonfat dry milk	Carl-Roth GmbH & Co.KG, Karlsruhe (D)
Nonidet P-40	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Opti-MEM®, reduced serum media	Gibco®, Darmstadt (D)
Penicillin-Streptomycin, liquid	Gibco®, Darmstadt (D)

Pertex mounting medium	Medite GmbH, Burgdorf, (D)
Phosphate buffered saline (PBS)	GE Healthcare, Munich (D)
PhosSTOP Phosphatase Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim (D)
Polybrene	Carl-Roth GmbH & Co.KG, Karlsruhe (D)
Ponceau S dye	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Potassium chloride	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Prestained Protein ladder Plus, PageRuler™	Thermo Fisher Scientific, Schwerte (D)
Prestained Protein ladder, PageRuler™	Thermo Fisher Scientific, Schwerte (D)
Propidiumiodid	AppliChem GmbH, Darmstadt (D)
Protease inhibitor cocktail tablets complete mini	Roche Diagnostics, Mannheim (D)
Proteinase K	Roche Diagnostics, Mannheim (D)
Puromycin	Life Technologies GmbH, Darmstadt (D)
RNase inhibitor RNaseOUT™	Life Technologies GmbH, Darmstadt (D)
RNaseZAP®	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Rneasy mini Kit	Qiagen GmbH, Hilden (D)
Saracatinib (AZD0530)	BioCat GmbH, Heidelberg (D)
Sodium chloride	Merck KgaA, Darmstadt (D)
Sodium dodecyl sulfate 20 % (w/v) solution	AppliChem GmbH, Darmstadt (D)
Sucrose	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Super Signal West Femto Chemiluminescent Substrate	Thermo Fisher Scientific, Schwerte (D)
Super Signal West Pico Chemiluminescent Substrate	Thermo Fisher Scientific, Schwerte (D)
Taq DNA Polymerase BioTherm™	Ares Bioscience GmbH, Köln (D)
Temgesic®	Essex Pharma GmbH, Munich (D)
Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories GmbH , Munich (D)
Toluidine blue	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Transfection Reagent X-tremeGENE for siRNA	Roche Diagnostics, Mannheim (D)
Triton X-100	Merck KGaA, Darmstadt (D)
Trizma® base	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Trypan blue solution 0.4 %	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Trypsin, 0.05 % with EDTA	PAA Laboratories GmbH, Cölbe (D)
Tween 20	Merck KGaA, Darmstadt (D)
Vectashield mounting medium for fluorescence	Vectashield, Loerrach (D)
ViraPowerTM Lentiviral Expression System	Life Technologies GmbH, Darmstadt (D)
Water, Aqua ad iniectabilia	AlleMan Pharma GmbH, Rimbach (D)

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	Life Technologies GmbH, Darmstadt (D)
Xylol	Merck KGaA, Darmstadt (D)
β-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim (D)

2.1.2 Consumables

Product	*Company*
Amersham Hybond™ ECL™ Nitrocellulose Membrane	GE Healthcare Lifescience, Freiburg (D)
Amersham Hyperfilm ECL	GE Healthcare Lifescience, Freiburg (D)
BD BioCoat Collagen I – 24-well Plate	BD Bioscience, Heidelberg (D)
BD BioCoat Collagen I – 96-well Plate	BD Bioscience, Heidelberg (D)
BD BioCoat™ Matrigel™ Invasion Chambers	BD Bioscience, Heidelberg (D)
Cell Scraper	SARSTEDT AG & Co., Nümbrecht (D)
Combitips® for Multipette® 5 ml	Eppendorf AG, Hamburg (D)
Corning®DeckWorks® Pipette tips, 10 µl	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Corning®DeckWorks® Pipette tips, 1000 µl	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Corning®DeckWorks® Pipette tips, 20 µl	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Corning®DeckWorks® Pipette tips, 200 µl	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Coverslips	Carl Roth GmbH & Co.KG, Karlsruhe (D)
Cryotubes	A. Hartenstein, Würzburg (D)
Embedding cassettes	A. Hartenstein, Würzburg (D)
MicroAmp® Optical 96-Well Reaction Plate	Life Technologies GmbH, Darmstadt (D)
Microscope Slide	Thermo Fisher Scientific, Schwerte (D)
Mini-PROTEAN Tetra Electrophoresis System	Bio-Rad Laboratories GmbH , Munich (D)
Minisart filters pore size 0.2 µm	Sartorius AG, Göttingen (D)
PCR tube strips 0.2 ml	Eppendorf AG, Hamburg (D)
Pipettes, Pasteur glass 3.2 ml	Carl-Roth GmbH & Co.KG, Karlsruhe (D)
Pipettes, serological CELLSTAR® 10 ml	Greiner Bio-One, Frickenhausen (D)
Pipettes, serological CELLSTAR® 25 ml	Greiner Bio-One, Frickenhausen (D)
Pipettes, serological CELLSTAR® 5 ml	Greiner Bio-One, Frickenhausen (D)
Pipettes, serological CELLSTAR® 50 ml	Greiner Bio-One, Frickenhausen (D)
PowerPac Basic Power Supply	Bio-Rad Laboratories GmbH , Munich (D)
Reaction tubes 1.5 ml	Eppendorf AG, Hamburg (D)
Reaction tubes 2 ml	Eppendorf AG, Hamburg (D)

Reaction tubes Falcon™ Blue Max 15 ml	BD Bioscience, Heidelberg, (D)
Reaction tubes Falcon™ Blue Max 50 ml	BD Bioscience, Heidelberg, (D)
Safe seal microtubes	Eppendorf AG, Hamburg (D)
Serological Pipettes cellstar®, 10 ml	Greiner Bio-One, Frickenhausen (D)
Serological Pipettes cellstar®, 2 ml	Greiner Bio-One, Frickenhausen (D)
Serological Pipettes cellstar®, 25 ml	Greiner Bio-One, Frickenhausen (D)
Serological Pipettes cellstar®, 5 ml	Greiner Bio-One, Frickenhausen (D)
Syringe, single-use 20 ml	B. Braun Melsungen AG, Melsungen (D)
Tissue culture dish	TPP, Trasadingen (CH)
Tissue culture flasks (25 cm^2, 75 cm^2)	TPP, Trasadingen (CH)
Tissue Culture Test Plates (96-well, 24-well, 6-well)	TPP, Trasadingen (CH)
Whatman filter paper	Neolab, Heidelberg (D)
Wide mini Sub Set GT electrophoresis system	Bio-Rad Laboratories GmbH , Munich (D)

2.1.3 Equipment

Product	*Company*
Accu-jet® pro	Brand GmbH + CO KG, Wertheim (D)
ASYS Expert Plus Microphotometer 8 Kanal	Biochrom AG, Berlin (D)
Axio Observer Z1	Carl Zeiss AG, Jena (D)
Axiocam ICm1	Carl Zeiss AG, Jena (D)
Axiovert 135	Carl Zeiss AG, Jena (D)
Axiovert 25	Carl Zeiss AG, Jena (D)
Cat SRX 101 A development machine	Konica minolta, Langenhagen (D)
Centrifuge 5415D	Eppendorf AG, Hamburg (D)
Centrifuge 5417R	Eppendorf AG, Hamburg (D)
Concentrator plus	Eppendorf AG, Hamburg (D)
Dako Autostainer Universal Staining System	Dako Deutschland GmbH, Hamburg (D)
Dispenser Multipette® plus	Eppendorf AG, Hamburg (D)
Ebox VX2 gel documentation system	Peqlab Biotechnology GmbH, Erlangen (D)
Electrophoresis Transfer Cell mini Trans-Blot®	Bio-Rad Laboratories GmbH , Munich (D)
Eppendorf Mastercycler gradient	Eppendorf AG, Hamburg (D)
Eppendorf research pipettes	Eppendorf AG, Hamburg (D)
Filter set 38 Endow GFP shift free	Carl Zeiss AG, Jena (D)
Filter set 43 Cy3, d=25 shift free	Carl Zeiss AG, Jena (D)

Filter set DAPI	Carl Zeiss AG, Jena (D)
Filterset 38 Endow GFP	Carl Zeiss AG, Jena (D)
Filterset 43 Cy3	Carl Zeiss AG, Jena (D)
Freezer -20 °C Liebherr Comfort	Liebherr GmbH, Biberach an der Riss (D)
Freezer -20 °C, economic froster	Bosch GmbH, Stuttgart (D)
Freezer -80 °C HFC86-360	Heraeus Instruments, Osterode (D)
Freezer -80 °C Sanyo Ultra-low temperature chest freezer MDF-594	Panasonic, San Diego (USA)
Freezing container NALGENE™ Cryo 1 °C	Thermo Fisher Scientific, Schwerte (D)
Fridge +4 °C Liebherr Premium	Liebherr GmbH, Biberach an der Riss (D)
Gel documentation system	Vilber Lourmat, Eberhardzell (D)
GFL incubation waterbath	GFL GmbH, Burgwedel (D)
Glass coverslipper Promounter RCM2000	Medite GmbH , Burgdorf (D)
Haereus Hera Safe CO2 incubator	Thermo Fisher Scientific, Schwerte (D)
Heating block Thermomixer® comfort 1.5 ml	Eppendorf AG, Hamburg (D)
Heating block ThermoStat plus 1.5 ml	Eppendorf AG, Hamburg (D)
Heating oven type BE400	Memmert, Schwabach (D)
Heating plate HP 30 digital IKATHERM	IKA®-Werke GmbH & CO. KG, Staufen (D)
Heracell 150i CO2 incubator	Thermo Fisher Scientific, Schwerte (D)
Hood Uniflow UVUB 1800	UniEquip, Planegg (D)
Hotplate stirrer IKAMAG® RCT	IKA® Werke GmbH und Co.KG, Staufen (D)
Ice machine	ZIEGRA Eismaschinen GmbH, Hannover (D)
IKA® MS1 minishaker	IKA® Werke GmbH und Co.KG, Staufen (D)
Ikamage® RCT magnetic stirrer	IKA® Werke GmbH und Co.KG, Staufen (D)
Incubator innova CO-170	New Brunswick Sci., Edison (NJ, USA)
Incubator shaker Model G25	New Brunswick Sci., Edison (NJ, USA)
Lab centrifuge 4K15	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Laboratory balance BP 310 S	Sartorius AG, Göttingen (D)
Leitz Labovert FS	Leica Microsystems GmbH, Wetzlar (D)
Liquid Nitrogen tank MVE TEC 3000 (1500 series)	Chart MVE BioMedical GmbH, Wuppertal (D)
Magnetic stir bars, various sizes	NeoLab, Heidelberg (D)
Magnetic stirrer MR2000	Heidolph Instruments, Schwabach (D)
Manual Tissue Arrayer	Beecher Instruments Inc., Sun Prairie (USA)
Micro Writer	Thermo Fisher Scientific, Schwerte (D)
Microm HM 335 E	Thermo Fisher Scientific, Schwerte (D)

Microplate Reader Model 680	Bio-Rad Laboratories GmbH , Munich (D)
Microscope EVOS xl	Life Technologies GmbH, Darmstadt (D)
Microtome plade	Pfm medical AG, Köln (D)
Microwave Privileg 1034HGD	Otto, Hamburg (D)
Miniprotean system 3 cell	Bio-Rad Laboratories GmbH , Munich (D)
Mini-PROTEAN Tetra Electrophoresis System	Bio-Rad Laboratories GmbH , Munich (D)
Mitsubishi P95 printer	Mitsubishi Electric, Ratingen (D),
Nanodrop ND 2000c	Peqlab Biotechnology GmbH, Erlangen (D)
Neubauer counting chamber	Carl-Roth GmbH & Co.KG, Karlsruhe (D)
Paraffin Embedding System Medite TBS 88	Medite GmbH, Burgdorf, (D)
pH-Meter (pH 211)	HANNA Instruments GmbH, Kehl am Rhein (D)
Pipettor pipetus-akku®	Hirschmann Laborgeräte, Eberstadt (D)
Power supply PowerPac 300	Bio-Rad Laboratories GmbH , Munich (D)
PowerPac Basic Power Supply	Bio-Rad Laboratories GmbH , Munich (D)
Pressure cooker	WMF, Geislingen/Steige (D)
Rocker table Rocky®	Fröbel Labortechnik, Lindau (D)
Scales Sartorius universal	Sartorius AG, Göttingen (D)
Shandon Excelsior ES	Thermo Fisher Scientific, Schwerte (D)
Spectrophotometer NanoDrop® ND-1000	Thermo Fisher Scientific, Schwerte (D)
Spectrophotometer UV/VIS Novaspec II	Pharmacia LKB, Uppsala (S)
Stuart Scientific SD Rocking Platform STR	Keison products, Chelmsford (GB)
Thermomixer compact	Eppendorf AG, Hamburg (D)
Tissue Cool Plate COP 20	Medite GmbH, Burgdorf, (D)
Tissue Floatation Baths TFB 35	Medite GmbH, Burgdorf, (D)
Vacuum Concentrator plus	Eppendorf AG, Hamburg (D)
Vortexer MS1 minishaker	IKA®-Werke GmbH & CO. KG, Staufen (D)
Vortexer, VF 2	IKA®-Werke GmbH & CO. KG, Staufen (D)
Water bath shaker #1083	GFL GmbH, Burgwedel (D)
Wide mini Subset GT electrophoresis system	Bio-Rad Laboratories GmbH , Munich (D)

2.1.4 Kits

High capacity cDNA Reverse Transcription Kit	Invitrogen™, Darmstadt (D)
Pierce® BCA Protein Assay Kit	Perbio Science Deutschland GmbH, Bonn (D)
QIAprep Spin Maxiprep Kit	Qiagen GmbH, Hilden (D)
QIAprep Spin miniprep Kit	Qiagen GmbH, Hilden (D)

QIAquick Gel Extraction Kit	Qiagen GmbH, Hilden (D)
QIAquick PCR Purification Kit	Qiagen GmbH, Hilden (D)
ViraPower™ Lentiviral Expression Systems	Invitrogen™, Darmstadt (D)

2.1.5 Antibodies

Table 3: Primary antibodies

Product	*Dilution/Application*	*Company*
Anti-β_1-integrin	IHC (1:50) WB (1:1000)	BD Transduction Laboratories, Heidelberg (D)
Anti- β_1-integrin Clone P5D2	Blocking (2.5 µg/1x10^6 cells)	R&D Systems, Wiesbaden-Nordenstadt (D)
Anti-CD34 (QBEnd/10)	IHC (1:50)	Leica Biosystems GmbH, Nussloch (D)
Anti-CD31	IHC (1:50)	Abcam plc, Cambridge (UK)
Anti-COL5A1 (C-5)	IF (1:100)	Santa Cruz, Heidelberg (D)
Anti-COL5A1 (H-200)	WB (1:500) IHC (1:150)	Santa Cruz, Heidelberg (D)
Anti-FAK	WB (1:500)	BD Transduction Laboratories, Heidelberg (D)
Anti-GAPDH	WB (1:5000)	Santa Cruz, Heidelberg (D)
Anti-PAX	WB (1:1000)	New England Biolabs, Frankfurt (D)
Anti-pFAK-Y397	IF (1:100)	Santa Cruz, Heidelberg (D)
Anti-pFAK-Y397	WB (1:750)	BD Transduction Laboratories, Heidelberg (D)
Anti-pFAK-Y576/577	WB (1:750)	New England Biolabs, Frankfurt (D)
Anti-pFAK-Y861	IF (1:100)	Santa Cruz, Heidelberg (D)
Anti-pPAX-Y118	IF (1:100)	Santa Cruz, Heidelberg (D)
Anti-pPAX-Y118	WB (1:1500)	GeneTex Inc., Eching (D)
Anti-Vinculin (Clone hVIN-1)	IF (1:200)	Sigma-Aldrich Chemie GmbH, Steinheim (D)
Anti-αSMA	WB (1:2000)	Abcam plc, Cambridge (UK)
Anti-αSMA (HHF35)	IHC (1:100)	Dako Deutschland GmbH, Hamburg (D)

Table 4: Secondary antibodies

Product	*Dilution/Application*	*Company*
Alexa Fluor 488 goat antimouse IgG	IF (1:200)	Life Technologies GmbH, Darmstadt (D)
Alexa Fluor 546 goat antimouse IgG	IF (1:200)	Life Technologies GmbH, Darmstadt (D)
Biotin-labeled goat anti-mouse IgG	IHC (Ready to use)	KPL, Inc., Gaithersburg (USA)
Biotin-labeled goat anti-rabbit IgG	IHC (Ready to use)	KPL, Inc., Gaithersburg (USA)
ECL™ anti-mouse IgG	WB (1:2000)	GE Healthcare, Munich (D)
ECL™ anti-rabbit IgG	WB (1:2000)	GE Healthcare, Munich (D)

2.1.6 Buffers

Blotting buffer

TRIS base	48 mM
Glycine	39 mM
SDS	0.025 %
Methanol	20 %

Cell cycle Solution I

NaCl	10 mM
Na-citrate	40 mM
Nonidet P-40	0.003 %
RNase A	10 mg/l
Propidium iodide	2.5 %

Cell cycle Solution II

Citric acid	70 mM
Sucrose	0.25 M
Propidium iodide	4 %

Citrate buffer

Citric acid	10 mM
pH 6	

Lämmli 5X

TRIS	312.5 mM
Glycerol	40 %
SDS	10 %
Bromophenol blue	0.005 %
β-Mercaptoethanol	20 %

Ponceau S

Ponceau S	1 g
Acetic acid	1 %

RIPA buffer

TRIS pH 7.4	20 mM
NaCl	150 mM
EDTA	1mM
EGTA	1mM
Nonidet P-40	1 %
Deoxycholic Acid	0.5 %

RLN

TRIS-HCl	50 mM
NaCl	140 mM
$MgCl_2$	1.5 mM
Nonidet P-40	0.5 % (v/v)
pH 8.0	

Running buffer

TRIS base	12.5 mM
Glycine	9.6 mM
SDS	0.05 %

Running gel buffer 4X

TRIS-HCl	1.5 M
pH 8.8	

Stacking gel buffer 4X

TRIS-HCl	0.5 M
pH 6.8	

Stripping buffer

MetOH	10 %
Acetic acid	10 %

TBS 10X for Immunofluorescence

TRIS base	500 mM
NaCl	1380 mM
KCl	27 mM
pH 7.6	

TBS 10X for Western Blot

TRIS base	200 mM
NaCl	1360 mM NaCl
pH 7.6	

X-gal staining solution

NaCl	135 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
K_2HPO_4,	1.4 mM
$K_3Fe(CN)_6$	2.5mM
$K_4Fe(CN)_6$	2.5 mM
$MgCl_2$	1 mM
Na-Deoxycholat	0.0005 % (v/v)
NP40	0.001 % (v/v),
pH 7,1	

2.1.7 Tissue samples

Table 5: Patient characteristics

	Age	Gender	Grading	T	N	M
HD-45-1	63	Male	3	2	0	0
HD-176-1	66	Male	2	3	1a	0
HD-151	55	Male	2	3	1b	0
HD-197	62	Male	unknown	3	1b	0
HD-231-1	84	Male	2	3	0	0
HD-254-1	N/A	Male	2	3	1	0
HD-257-1	66	Female	2	3	1	0
HD-267	61	Male	2	3	1	0
HD-314-1	60	Female	2	3	1	0
HD-334-1	63	Male	2	4	1	0
HD-338-1	67	Female	3	3	1	1
HD-367-3	N/A	Male	2	3	1	0
HD-408-1	50	Female	2	3	1	0
HD-422-1	63	Female	2	3	1	0
HD-436-1	65	Female	3	3	1	1
HD-451-1	62	Male	unknown	3	1	0
HD-479	51	Male	3	3	1	0
HD-498	58	Male	2	3	1	0
HD-505-1	67	Male	2	3	1	0
HD-514-1	47	Male	2	3	1	0
HD-535-1	49	Male	2	3	0	0
HD-540	65	Male	3	3	0	0

HD-607	53	Female	2	3	0	0
HD-623	68	Female	3	3	1	0
HD-654	63	Male	2	3	1	0
HD-660	60	Female	2	3	1	0
HD-668	N/A	Female	2	3	1	0
HD-689	N/A	Male	2	3	1	0
HD-692	44	Female	2	3	1	0
HD-702	75	Male	2	3	0	0
HD-746	N/A	Female	3	3	1	0
HD-751-1	72	Female	3	3	1	0
HD-755-2	69	Male	2	3	1	0
HD-775-1	62	Female	3	3	1	0
HD-784-1	72	Male	2	3	1	0
HD-798-1	66	Female	3	3	1	0
HD-801-1	N/A	Male	2	3	1	0
HD-831-1	N/A	Male	2	3	1	0
HD-841	N/A	Male	3	3	1	0
HD-954-1	N/A	Female	1	3	1	0
HD-973-1	N/A	Female	3	3	1	0
HD-997-1	70	Female	2	3	0	0
HD-1007-1	55	Male	3	3	1	0
HD-1018	81	Male	2	3	1	0
HD-1035-1	70	Male	2	3	1	0
HD-1036-1	N/A	Male	2	3	1	0
HD-1050-1	48	Male	2	3	1	0
HD-1073-3	70	Female	2	3	1	0
HD-1148	N/A	Female	3	3	0	0
HD-1169-1	49	Male	3	3	1	0

2.1.8 Cell lines

Table 6: Source, culture properties and mutation status of the cell lines used

AsPC-1

Organism	Homo sapiens, 62 years, female, caucasian
Tissue	pancreas; derived from metastatic site: ascites
Origin	ATCC: The Global Bioresource Center
Culture Properties	adherent
Disease	adenocarcinoma
Mutations	*P53-/-* *KRAS* activating mutation *CDKN2A* frameshift mutation

Capan-1

Organism	Homo sapiens, 40 years, male, caucasian
Tissue	pancreas; derived from metastatic site: liver
Origin	ATCC: The Global Bioresource Center
Culture Properties	adherent
Disease	adenocarcinoma
Mutations	*P53* inactivation mutation *KRAS* activation mutation *CDKN2A* homozygous deletion

PANC-1

Organism	Homo sapiens, 56 years, male, caucasian
Tissue	pancreas/duct
Origin	*ATCC*: The Global Bioresource Center
Culture Properties	adherent
Disease	epithelioid carcinoma
Mutations	*P53* inactivation mutation *KRAS* activation mutation *CDKN2A* homozygous deletion

SU.86.86

Organism	Homo sapiens, 57 years, female, caucasian
Tissue	pancreas; derived from metastatic site: liver
Origin	*ATCC*: The Global Bioresource Center
Culture Properties	adherent
Disease	ductal carcinoma
Mutations	*P53* inactivation mutation *KRAS* activation mutation *CDKN2A* homozygous deletion

HUVEC

Organism	Homo sapiens
Tissue	Umbilical vascular endothelium
Origin	Kindly provided from AG Kleeff, Department of Surgery, TUM
Culture Properties	adherent

Ehlers-Danlos fibroblasts (F011 and F057)

Organism	Homo sapiens Ehlers-Danlos syndrome, classic type
Tissue	Skin
Origin	Eurobiobank, Milan (I)
Culture Properties	adherent

Human dermal fibroblasts

Organism	Homo sapiens
Tissue	Skin
Origin	Isolated and kindly provided from AG Schlegel, Institute of Pathology, TUM
Culture Properties	adherent

Pancreatic stellate cells

Organism	Homo sapiens
Tissue	pancreas
Origin	Isolated and kindly provided from AG Erkan, Department of Surgery, TUM
Disease	pancreatic adenocarcinoma
Culture Properties	adherent

HEK293T

Organism	Homo sapiens
Tissue	Human embryonic kidney
Origin	Kindly provided from AG Krüger, IEOT, TUM
Culture Properties	adherent

2.1.9 Database and softwares

Product	*Company*
Adobe Acrobat 9 Pro	Adobe Systems GmbH, Munich (D)
AxioVision	Carl Zeiss AG, Jena (D)
Endnote X6	Endnote, Carlsbad (USA)
Ensembl	http://www.ensembl.org/index.html
Gimp 2.0	http://www.gimp.org/
GraphPad PRISM	GraphPad Software, Inc., La Jolla, CA, (USA)
Human Protein Reference Database	http://www.hprd.org/index_html
Image J	National Institutes of Health
Microsoft Office 2007, 2010	Microsoft (Redmond, USA)
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
OlyVIA	Olympus Deutschland GmbH, Hamburg (D)
Online Mendelian Inheritance in Man	http://omim.org/
UniProtKB	http://www.uniprot.org/

2.1.10 Primer pairs

Table 7: Sequences of applied primers

Primer for mycoplasma test:

Primer supermix 1

Myco-Fwd 1	5'-ACA CCA TGG GAG TTG GTA AT-3'	5 µM
Myco-Fwd 1t	5'-ACA CCA TGG GAG CTG GTA AT-3'	5 µM
Myco-Rev 1tt	5'-CTT CTT CGA CTT TCA GAC CCA AGG CAT-3'	2.5 µM
Myco-Rev 1	5'-CTT CAT CGA CTT TCA GAC CCA AGG CAT-3'	2.5 µM
Myco-Rev 1cat	5'-CCT CAT CGA CTT TCA GAC CCA AGG CAT-3'	2.5 µM
Myco-Rev 1ac	5'-CTT CAT CGA CTT CCA GAC CCA AGG CAT-3'	2.5 µM

Primer supermix 2

Myco-Fwd 2a	5’-ATT CTT TGA AAA CTG AAT-3’	2.5 µM
Myco-Fwd 2	5’-GTT CTT TGA AAA CTG AAT-3’	2.5 µM
Myco-Fwd 2cc	5’-GCT CTT TCA AAA CTG AAT-3’	2.5 µM
Myco-Rev 2ca	5’-GCA TCC ACC ACA AAC TCT-3’	2.5 µM
Myco-Rev 2	5’-GCA TCC ACC AAA AAC TCT-3’	2.5 µM
Myco-Rev 2at	5’-GCA TCC ACC AAA TAC TCT-3’	2.5 µM

qRT-PCR Primer

Eukaryotic 18S rRNA Endogenous Control	Applied Biosystems®, Darmstadt (D)
Collagen type V	Universal ProbeLibrary - Roche Applied Science
	Forward: 5’-GGA CCC CAA TCC AGA TGA ATA-3’
	Reverse: 5’-AGG GGT ATT AGT AGT AAT AGG TCT CA-3’

2.1.11 SiRNA/shRNA

Table 8: Sequences of applied siRNA and shRNA

AllStars Negative Control siRNA	Not available	Qiagen GmbH, Hilden (D)
Hs_COL5A1_1 FlexiTube siRNA	5’-CAC CTT GAT CCT CGA CTG TAA-3’	Qiagen GmbH, Hilden (D)
shNT	Not available	Sigma-Aldrich Chemie GmbH, Steinheim (D)
shCOL5A1 (#10) TRCN0000082810	5’-CCGGGCTCTATTGGATTCCCTGGATCTCGAGATCCAGGGAATCCAATAGAGCTTTTTG-3’	Sigma-Aldrich Chemie GmbH, Steinheim (D)
shCOL5A1 (#11) TRCN0000082811	5’-CCGGCCGTATGATGACCTCACCTATCTCGAGATAGGTGAGGTCATCATACGGTTTTTG-3’	Sigma-Aldrich Chemie GmbH, Steinheim (D)

2.1.12 Plasmids for lentiviral transduction

Plasmid	*Function*	*Company*
pLP1	Packaging plasmid (gag, pol)	Life Technologies GmbH, Darmstadt (D)
pLP2	Packaging plasmid (rev)	Life Technologies GmbH, Darmstadt (D)
pLP/VSVG	Packaging plasmid (env from VSVG)	Life Technologies GmbH, Darmstadt (D)
pLenti6/V5lacZ	lacZ-transgen	AG Krüger, IEOT, TUM

2.2 Methods

2.2.1 Tissue collection

Tissue samples were obtained from patients who underwent pancreatic resections for PDAC at the Technische Universität München, Germany or the Ruprecht-Karls-Universität Heidelberg, Germany. After surgical removal, tissue samples were fixed in 4 % buffered formalin and embedded in paraffin after 24 hrs. Tissue arrays were obtained with a manual tissue arrayer (Beecher Instruments Inc., USA).

2.2.2 Ethics

The use of human material for the analysis was approved by the local ethics committee of the Technische Universität München or the University of Heidelberg, Germany, and written informed consent was obtained from all patients. Animal experiments were conducted in compliance with the guidelines of the "Tierschutzgesetz des Freistaates Bayern" and approved by the "Regierung von Oberbayern" with the permission number: 55.2-1-54-2532-42-13.

2.2.3 Hematoxylin and eosin stain

Paraffin fixed tissues were dewaxed in xylol and rehydrated in a graded alcohol series. Then sections were washed in distilled water and stained for 5 min with hematoxylin for nuclear staining. The slides were washed with tap water for 5 min to obtain differentiation. Eosinophilic structures were stained for 5 min with eosin. Subsequently, the slides were washed in water and dehydrated in alcohol and xylene. To protect the tissue, coverslips were coverslips were applied with a drop of mounting medium.

2.2.4 Immunohistochemistry

For immunohistochemistry the tissues were dewaxed and rehydrated as described above. Heat-induced antigen retrieval was done in a pressure cooker with citrate buffer for 10 min. Further staining was performed either manually (CD34) or with the DAKO Autostainer. Endogenous peroxidase was blocked in 3 % H_2O_2 in methanol for 10 min (anti-CD34, anti-CD31) or universal block for 20 min (anti-β_1-integrin, anti-Col V). Nonspecific antibody binding was blocked by 3 % normal goat serum for 30 min. Primary antibody was added over night at 4 °C (anti-CD34) or 1 hr at RT (anti-β_1-integrin, anti-Col V, anti-CD31). Slides were washed three times in TBS or Dako buffer and the corresponding biotinylated secondary antibody was added for 30 min at room temperature (RT). Slides were washed again three times with TBS before streptavidin-horseradish peroxidase was added for 30 min at RT. After another washing step the color reaction was performed using diaminobenzidine (DAB) as chromogen. The color reaction was observed under the microscope and stopped with distilled water. After DAB staining, counterstaining with hematoxylin for 10 min was done followed by differentiation under running tap

water. After the staining procedure the slides were dehydrated in ethanol and xylene. To protect the tissue, coverslips were applied with a drop of mounting medium.

2.2.5 Propagation of mammalian cell lines

2.2.5.1 Pancreatic cancer cells

Pancreatic cancer cells lines AsPC-1, Capan-1, PANC-1 and SU.86.86 were maintained in high glucose DMEM GlutaMAX™ medium containing 10 % FBS (heat denatured at 56 °C for 30 min) and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2. Passaged cells were kept in 75 cm^2 tissue culture flasks. Cells were tested for mycoplasma contamination by polymerase chain reaction (PCR) detection (see 2.2.5.10), shortly after thawing.

2.2.5.2 Pancreatic stellate cells

PSC were isolated and characterized as previously described in detail (Koninger, Giese et al. 2004) and cultured in a 1:1 dilution of Ham's F-12 nutrient medium and low-glucose DMEM supplemented with 1 % antibiotics, 1 % amphotericin B and 20 % FBS at standard conditions.

2.2.5.3 Human umbilical vein endothelial cells (HUVEC)

HUVEC cells were cultured in endothelial growth medium supplemented with 1 % penicillin/streptomycin at standard conditions.

2.2.5.4 HeLa cells

The cervical cancer cell line HeLa was maintained in DMEM GlutaMAX™ medium containing 10 % FBS (heat denatured at 56 °C for 30 min) and 1 % penicillin/streptomycin. These cell lines were used for protein extractions as a positive control.

2.2.5.5 Human dermal fibroblast and fibroblast of Ehlers Danlos-syndrome

Normal human dermal fibroblasts (HDF) and dermal fibroblasts of patients suffering from the Ehlers-Danlos syndrome (F011 and F057) were maintained in high glucose DMEM GlutaMAX™ medium containing 10 % FBS (heat denatured at 56 °C for 30 min) and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2.

2.2.5.6 Cell splitting

Cells at 80 % confluence were washed with PBS and detached by trypsinisation at 37 °C. The reaction was stopped with standard culture medium and cells were transferred into a new flask at the appropriate concentration.

2.2.5.7 Cell counting

Cells were counted with a hemocytometer (improved Neubauer hemocytometer). Cell suspensions of 15 µl were mixed with trypane blue 1:1 to distinguish dead from living cells.

2.2.5.8 Freezing cell lines

Cells at 80 % confluence in 75 cm^2 flasks were trypsinized, resuspended in complete medium and centrifuged at 1000 rpm for 5 min. Cell pellets were resuspended in 2 ml complete medium containing 10 % DMSO. 1 ml aliquots were transferred into cryovials, frozen slowly at -80 °C in a cryo freezing container for 24 hrs and then moved into liquid nitrogen for long term storage.

2.2.5.9 Thawing frozen cell lines

After cells were thawed quickly in a 37 °C water bath, they were moved into 15 ml falcon tubes and 10 ml pre-warmed medium was added slowly. Cells were then centrifuged at 1000 rpm for 5 min to remove toxic DMSO and the pellet was resuspended in complete medium and transferred to a culture flask.

2.2.5.10 Nested PCR for mycoplasma contamination

To exclude mycoplasma contamination, nested PCR for the detection of contamination with mycoplasma was used (Uemori 1992). Therefore, 1 ml of medium was taken from an 80 % confluent cell culture flask. The medium was diluted 1:10, incubated for 5 min at 95 °C and subsequent centrifuged shortly. The supernatant was used directly as template for the PCR.

For the first PCR, a 25 µl reaction was set as follows:

dH_2O	15 µl
10X buffer (15 mM Mg^{2+})	2.5 µl
dNTPs (10 mM each)	0.5 µl
Primer supermix 1	1.0 µl
Taq polymerase	1.0 µl
Supernatant	5.0 µl

The amplification parameters were set as follows:

94 ° C	4 min initial denaturation
35 cycles with:	
95 °C	30 sec denaturation
55 °C	2 min annealing
72 °C	1 min elongation
72 °C	7 min final elongation

For the second PCR a 25 µl reaction was set as follows:

dH_2O	19 µl
10X buffer (15 mM Mg^{2+})	2.5 µl
dNTPs (10 mM each)	0.5 µl
Primer supermix 2	1.0 µl
Taq polymerase	1.0 µl
Supernatant	1.0 µl

The amplification parameters were set as follows:

94 ° C	4 min initial denaturation
30 cycles with:	
95 °C	30 sec denaturation
55 °C	2 min annealing
72 °C	1 min elongation
72 °C	7 min final elongation

The amplification product was visualized by gel electrophoresis on 1 % agarose gel in TBE containing 0.5 µg/ml ethidium bromide. Gel images were taken with a gel documentation system.

2.2.5.11 Preparation of conditioned medium

For the preparation of conditioned medium (CM), cells were incubated for 24 hrs with their respective standard medium. Medium was then collected and centrifuged at 12 000 rpm for 5 min to remove cell debris.

2.2.6 Collagen type V coating

The wells of 6-, 24- or 96-well plates were coated with Col V dissolved in acetic acid, to give a final coating concentration of 10 µg/cm^2. Col V was allowed to be adsorbed overnight at 37 °C, the wells were then washed twice with PBS to remove unbound protein and blocked for 30 min at RT by adding of 0.1 % heat-denatured (85 °C for 10 min) BSA in PBS. The plates were finally washed three times with sterile PBS, dried under a laminar flow hood and sterilized by UV exposure for 20 min. Glass coverslips for immunofluorescence with a size of 11 mm were coated following the same protocol after being laid into the wells of a 24-well plate.

2.2.7 Immunofluorescence

Cells were plated onto 11 mm coated coverslips placed into 24-well plates and grown in media containing 10 % FBS for 24 hrs. Cells were then washed twice with PBS, fixed with methanol for 5 min at RT, washed twice with PBS and once with TBS buffer (50 mM TRIS, 138 mM NaCl, 2.7 mM KCl, pH 7.6), permeabilized with Triton X-100 (0.25 % v/v in TBS) for 5 min, before being washed again twice

with TBS. Nonspecific antibody binding was blocked by incubation of the coverslips for 1 hr with blocking solution (TBS containing 10 % goat serum and 0.1 % Triton X-100). Then, incubation with the primary antibody diluted in blocking solution was performed over night at 4 °C in a humidified chamber. After washing three times with TBS, slides were incubated with the Alexa Fluor conjugated secondary antibody (goat anti-mouse or anti-rabbit, 1:200) in blocking solution for 1 hr at RT. Slides were then washed again three times before being counterstained with Hoechst 33342 (0.5 µg/ml) for 5 min, briefly washed with TBS, covered with anti-fade mounting medium and placed onto microscope slides. Slides were examined under a fluorescence microscope (Carl Zeiss AG, Jena, Germany) and images were obtained with the Zeiss Axiovision software.

2.2.8 Protein extraction from cells

Culture medium was removed and cell monolayers were rinsed twice with cold PBS and then scraped in cold lysis buffer (20 mM TRIS pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Nonidet P-40, 0.5 % deoxycholic acid) supplemented with commercial protease and phosphatase inhibitor cocktails. After 15 min incubation on ice, cell lysates were passed through a 26G needle several times and cellular debris was removed by centrifugation at 12 000 rpm for 15 min at 4 °C. The protein concentration in the soluble fractions was determined using the BCA protein assay. Extracts were aliquoted and stored at -20 °C.

2.2.9 Protein quantification

The concentration of extracted proteins was determined to ensure equal loading in SDS-PAGE. To measure the concentration, the BCA Protein Assay Kit was used according to the manufacturer's instructions. A standard curve was prepared for each measurement. Absorbance was read at 550 nm with a microplate reader. A standard curve was drawn and protein concentration in unknown samples was extrapolated from the linear part of this curve.

2.2.10 Immunoblotting

Proteins in the needed concentration were mixed with 5X Laemmli (62.5 mM TRIS, 8 % glycerol, 2 % SDS, 0.001 % bromphenol blue, 4 % β-mercaptoethanol) and boiled for 5 min at 95 °C and 700 rpm. After boiling, extracts were cooled on ice and centrifuged shortly. Gel electrophoresis was conducted in running buffer (12.5 mM TRIS base, 9.6 mM glycine, 0.05 % SDS) in the Biorad mini-PROTEAN Electrophoresis 3 cell System at 25 mA. Proteins were then transferred with blotting buffer (48 mM TRIS base, 39 mM glycine, 20 % methanol, 0.025 % SDS, pH 8.3) to nitrocellulose membranes (Amersham Hybond ECL) applying 100 V for around 2.5-3 hrs, depending on protein size. Transfer was tested with Ponceau S-staining, which was removed again by rinsing the membranes with TBS-T

(20 mM TRIS, 136 mM NaCl, 0.1 % Tween-20, pH 6.8). Membranes were blocked either with 5 % BSA in TBS-T for 2 hrs at 37 °C or with 5 % nonfat dry milk in TBS-T at RT for 1 hr to block unspecific binding sites. The membrane was incubated overnight at 4 °C with the primary antibody, diluted in TBS-T with 3 % BSA. After three washes with TBS-T, horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in TBS-T with 5 % nonfat dry milk), was added for 1 hr at RT. The membrane was washed again three times with TBS-T before signals were visualized with a chemiluminescent substrate kit and developed using a light-sensitive imaging film. All the steps were performed with gentle agitation on a rotary shaker. Membrane stripping was performed using a stripping buffer (10 % methanol and 10 % acetic acid) for 30 min at RT.

2.2.11 Adhesion assay

Cells were starved for 24 hrs with medium supplemented with 1 % FBS. The next day, the cells were trypsinized, resuspended to a concentration of 150 000 cells/ml in medium with 1 % FBS. Then, 15 000 cells in a total volume of 100 µl were plated onto 96-well plates. Cells were let adhere for different time points, before the medium was removed and the wells were washed 3 times with PBS. Fixation of the adhered cells was performed with cold methanol for 5 min. After another washing step with water, cells were stained for 15 min with toluidine blue (0.5 % in water) and washed again. Excess water was aspirated and attached cells were lysed in 50 µl of 50 mM HCl diluted in 50 % ethanol. Absorption was measured at 620 nm using a microplate reader.

2.2.12 Proliferation assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay detects living cells according to their cellular metabolic activity *via* NAD(P)H-dependent cellular oxidoreductase enzymes, and reflects the number of viable cells through the activity of cellular enzymes that reduce the tetrazolium dye to its insoluble product formazan. This method can therefore be used to measure viability or proliferation (Mosmann 1983). Cells from 70-80 % confluent cultures were seeded into 96-well plates at their respective optimal numbers (AsPC-1: 7000, Capan-1: 8000, PANC-1: 5000 and SU.86.86 3000) in medium containing 10 % FBS. The cells were incubated up to 72 hrs. After incubation, MTT solution was added to the wells to a final concentration of 0.5 mg/ml and let be reduced to purple formazan in living cells for 3 hrs. Then, a solubilization solution (0.04 M HCl in isopropanol) was added to dissolve the purple formazan product. The absorbance of the solution was quantified by measuring at 560 nm.

2.2.13 Migration assay

Wound healing assay is a classical *in vitro* assay, in which the ability of cells to migrate into an open space is investigated (Lampugnani 1999). To determine the migration capability of different cell lines on different substrates, wound healing assays were performed. Cells were resuspended in 1 ml of complete medium and seeded onto the wells of a 24-well plate coated with Col V. BSA-coated wells were used as controls. The cells had to form a monolayer and were seeded at different numbers, depending on the cell line under analysis: 160 000 cells for PANC-1, 120 000 for SU.86.86, 250 000 for Capan-1, 100 000 for PSC and F057 and 120 000 for F011. The plate was incubated at 37 °C for at least 6 hrs to allow cell adhesion and afterwards complete media was changed with medium without FBS or 1 % FBS (for PSC and dermal fibroblasts). Cells were starved overnight and the next day the monolayer was scratched with a p10 pipette tip. Cells were washed twice with PBS to remove detached cells and pictures were taken at 0 hrs, 24 hrs and 48 hrs with an Axio Observer A.1 microscope (Carl Zeiss AG, Jena, Germany) at 10x magnification. The migration was evaluated analyzing the pictures with ImageJ analysis software. Cell-free areas were measured for the calculation of migration rates.

2.2.14 Drug treatment

Cells from 70-80 % confluent cultures were seeded into 96-well plates (cell counts were used as described in 2.2.12) in medium containing 10 % FBS. After 6 hrs incubation at standard conditions, medium was changed to medium containing 1 mM 5-fluorouracil (5-FU) or 10 µM gemcitabine (Gem). 72 hrs after seeding, MTT assay was performed as described in 2.2.12.

2.2.15 Apoptosis and cell cycle analysis

FACS analysis was done in cooperation with Dr. Wolfgang Beisker, Institute of Toxicology, Helmholtz Zentrum München, Neuherberg, Germany.

Apoptosis

For apoptosis measurements, 250 000 SU.86.86 cells were seeded per well of a 6-well plate and treated for up to 72 hrs with 10 µM Gem or 1 mM 5-FU. After incubation the cells were trypsinized, centrifuged and resuspended in PBS. Apoptotic cells were then measured using annexin V staining.

Cell cycle analysis

Before cell cycle analysis could be performed, cells had to undergo cell cycle synchronization. Therefore 100 000 cells were plated in 25 cm^2 culture flasks in complete medium and starved for 60 hrs in medium without FBS. Synchronized proliferation was started by adding of medium containing 10 % FBS (Pardee 1974). Cell cycle progression was analyzed in 12 hrs intervals up to 72 hrs. In detail, the

cells were trypsinized, centrifuged and the pellet was resuspended in Cell Cycle Solution I (10 mM NaCl, 40 mM Na-citrate, 0.003 % Nonidet P-40, 10 mg/l RNase A) containing 2.5 % propidium iodide. After incubation for 30 min at RT, Cell Cycle Solution II (70 mM citric acid, 0.25 M sucrose) with 4 % propidium iodide was added. In this procedure the cellular membrane and the cytoplasm are destroyed and nuclei are released in suspension (Nusse, Beisker et al. 1994). Samples could be stored at 4 °C for several weeks. The measurement of the DNA was performed by fluorescence activated cell sorting (FACS).

2.2.16 Inhibition of the ß$_1$-integrin signaling pathway

Two different known inhibitors of the β_1-integrin signaling pathway were used to block the Col V mediated signaling: a blocking antibody that blocks the binding of Col V on β_1-integrin (Wang, Bai et al. 2007) and a Src-inhibitor that inhibits the phosphorylation of FAK and therefore the downstream signaling of this pathway (Green, Fennell et al. 2009). Inhibition was tested in different PCC either in the presence or absence of Col V. For this purpose, $1x10^6$ cells were starved for 24 hrs in serum free medium and pre-incubated between 30-60 min at 37 °C with 2.5 µg anti-β_1-integrin (Clone P5D2) or 2 µM AZD. $1x10^6$ cells were seeded in the well of a 6-well plate and incubated for different time periods (AsPC-1 and Capan-1 15 min; SU.86.86 30 min) under standard conditions. After incubation, proteins were extracted in lysis buffer and lysates were frozen at -20 °C. Upregulation of phosphorylated proteins of the β_1-integrin-signaling pathway (pPAX-Y118, pFAK-Y397 and pFAK-Y576/577) and GAPDH (loading control) were detected by immunoblotting. For adhesion assays cells were preincubated with 2 µM AZD for 30 min and with 2.5 µg P5D2 per $1x10^6$ cells for 1 hr. For proliferation and migration assay inhibitors were added after cells were attached.

2.2.17 Transfection with siRNA

75 000 PSC or 150 000 HUVEC were plated in a 6-well plate and incubated over night to attach. The next day, medium was removed and cells were washed with PBS. Cells were starved for 2 hrs in reduced serum media (Opti-MEM®) until transfection. Transfection was performed with X-tremeGENE siRNA Transfection Reagent according to the user manual. For the transfection, 1 µg siRNA (5'-UUACAGUCGAGGAUCAAGGTG-3') and transfection reagent (5 µl) were first diluted in Opti-MEM®, then the solutions were mixed and incubated for 15 min at RT. After incubation, the mix was added dropwise to the cells. After 5 hrs, medium was replaced with standard medium. Efficiency of transfection was checked by immunoblotting.

2.2.18 Invasion assay

Chemoinvasion assay was performed using 24-well plates with 8 µm pore size polycarbonate membrane inserts coated with Matrigel™. Before use, inserts were rehydrated in serum free medium

for 2 hrs at 37 °C. Cells were then detached with trypsin and resuspended in medium containing 0.1 % FBS to a concentration of 50 000 cells/ml. 500 µl of the cell suspension (25 000 cells/well) were laid onto the insert, and the lower well was filled with 750 µl of the chemoattractant (CM from transfected and untransfected PSC). Cells were incubated for 24 hrs at 37 °C, 5 % CO_2. Cells and medium from the upper part of the insert were then removed using water humidified cotton swab and the insert with the cells that migrated through and adhered to the lower part of the membrane was fixed with methanol for 30 min. Cells were then stained with toluidine blue (2 % in water). Filters were dried 1 hr at 37 °C, removed from the well with a scalpel and mounted on microscope slides.

2.2.19 Morphometric analysis

Forty cases of human pancreatic cancer were stained in serial slides with Col V and CD34 as described in 2.2.4. Morphometric quantification of stained areas (vessels) was measured using the Definiens Enterprise Image Intelligence™ Suite software (Definiens AG, Munich, Germany) and correlation was calculated using GraphPad PRISM. Analysis was done in cooperation with Annette Feuchtinger, Research Unit Protein Science, Helmholtz Zentrum München, Neuherberg, Germany.

2.2.20 Tube-formation assay

Tube formation is an *in vitro* assay for angiogenic activities of endothelial cells that allows investigation of the influence of different conditions or compounds on angiogenesis. Two different models were used in this work. The formation of tube-like structures was tested with HUVEC using a collagen model and the standard matrigel model.

Collagen model

First, a layer of Col V or Col I was made. For the Col V layer, 10 µg/cm^2 were added onto a 96-well plate in a total volume of 100 µl and incubated at 37°C overnight. The second day, the layer was washed carefully with PBS and 25 000 cells were seeded on the layer. The cells were allowed to attach for 2 hrs and the medium was removed carefully. Then, the same amount of Col V diluted in EGM (endothelial growth medium) was added as the second layer and incubated for another 30 min. After another washing step, EBM (endothelial basal medium) containing 40 ng/ml bFGF (basic fibroblast growth factor) was added to allow tube formation.

Col I layers were produced in a similar way with a slightly different procedure. 100 µl EGM were supplemented with 0.5 % NaOH and 1.2 mg/ml Col I and the polymerization was 30 min according to a previously reported protocol (Montanez, Casaroli-Marano et al. 2002).

Matrigel model with siRNA

Matrigel™ was thawed at 4 °C for around 2 hrs before use; pipette tips and a 96-well plate were prechilled at -20 °C. 100 µl of Matrigel™ were added in 96-well plates and polymerized at 37 °C for 30 min. HUVEC with and without a transient knock-down of Col V (25 000 cells) were seeded on the Matrigel™ layer. Pictures were taken 16 hrs after plating the cells (according to Ponce 2009).

Matrigel model with CM

HUVEC were seeded on the Matrigel™ layer in conditioned or control media without FBS of PSC or PCC, respectively. The cells were incubated for 16 hrs before pictures were taken. CM containing cell secretions were collected from the cell lines after 24 hrs of culture. Untreated standard culture medium was used as controls.

2.2.21 Co-transfection for the production of lentiviral particles

To obtain a stable knock-down of Col V, lentiviral infection of cells was performed. Cotransfection of the plasmid containing the antibiotic resistance and the gene of interest as well as the packaging plasmids allow the insertion of the transgene in virions, which then accumulate in the supernatant after lysis of the packaging cell line. Packaging plasmids contain genes, which are important for the preparation of a non-replicative minimal lentivirus. The plasmid pLP1 contains the genes *gag* (viral core proteins required for forming the structure of the lentivirus) and *pol* (replication enzymes), pLP2 codes for *rev* (induces *gag* and *pol* expression) and pVSVG codes for the envelope vector (*env*). Plasmids were isolated with the Qiagen MaxiPrep Kit according to the manufacturer's instructions. Lentiviral particles were produced in human embryonic kidney stem cells (HEK293T) with the ViraPower™ Lentiviral Expression Systems according to the manufacturer's instruction. Co-transfection of the packaging cell line was conducted with Lipofectamine. Briefly, $5x10^6$ cells were plated in 10 cm culture dishes and incubated for 24 hrs at standard conditions. The next day, 6.6 µg pLP1, 5 µg pLP2, 3 µg pVSVG and 10 µg of the transferplasmid were mixed with Lipofectamine in Opti-MEM and incubated for 20 min at RT. The DNA-Lipofectamine-complex was then added dropwise on the cells. After 6 hrs Opti-MEM containing plasmids and Lipofectamine was replaced with standard complete medium. Lentiviral particles were harvested after 48 hrs. The supernatant was removed from the cells and sterile filtered with a 45 µm filter. Particles were used directly or stored at -80 °C. Plasmid preparations and S2-procedures of this experiment were performed in cooperation with M.Sc. Barbara Grünwald, Institute of Experimental Oncology, Prof. A. Krüger, Technische Universität München.

2.2.22 Transduction of pancreatic stellate cells

48 hrs before infection, PSC were seeded at a density of 70 % in 6-cm cell culture dishes, reaching a confluence of 80-90 % at the time point of transduction. Cells were washed with PBS before 1 ml of the lentiviral particles were applied to the cells. To enhance the efficiency of the infection 200 µg/ml polybrene was added. The cells were then incubated for 2 hrs at 37 °C while they were gently rotated every 15-20 min, before the virus was removed again. Efficiency of the knock-down was tested by TaqMan assay and immunoblotting.

2.2.23 Transduction of SU.86.86 for lacZ-tagging

For the detection of metastatic sites, cancer cells were tagged with lacZ *via* lentiviral transduction. The day before the transduction $2x10^5$ cells were seeded in 6-well plates. The next day, lentiviral particles were mixed with polybrene (8 µg/ml) and added to the cells. To test successful transduction, cells were stained with X-gal.

2.2.24 X-gal staining of adherent cells

To test the efficiency of the lacZ transduction, cells were stained with X-gal after 72 hrs. Briefly, cells were washed with PBS, fixed in fixing solution (2 % formaldehyde, 0.2 % glutaraldehyde in PBS), washed three times with PBS and stained with X-gal (Kruger, Sanchez-Sweatman et al. 1998). Evaluation was done with the help of a microscope.

2.2.25 X-gal staining of organs

To detect metastatic colonies of tumor cells, genetic labeling of the tumor cells is essential. This can be achieved through the stable insertion of the lacZ-gene, which codes for the enzyme galactosidase. The chromogene substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) can be metabolized through the galactosidase to galactose and 5-bromo-4-chloro-3-hydroxyindole, which then oxidizes to 5,5'-dibromo-4,4'-dichloro-indigo, an intensely blue product which is insoluble and stable for a long time (Kruger, Sanchez-Sweatman et al. 1998). To analyze the metastatic colony formation, X-gal staining of whole organs was performed. Therefore, organs were washed in PBS directly after necropsy and fixed for 1.5-2 hrs in fixing solution. Afterwards, organs were washed three times in PBS and stained with X-gal solution for 4-7 hrs at 37 °C and subsequently at 4 °C overnight. The next day, organs were washed with PBS and stored in fixing solution without glutaraldehyde. Blue stained cells on the liver surface were counted under the microscope.

2.2.26 Quantitative RT-PCR (TaqMan)

Knock-down efficiency of the different shRNA was validated *via* qRT-PCR. Sequence specific DNA probes were purchased from Roche (mRNA targets) or Applied Biosystems® (internal 18S rRNA standard), respectively. RNA isolation was performed with TRIzol according to the manufacturer's instructions. Reverse transcription was performed with the High capacity cDNA Reverse Transcription Kit using 1 µg of RNA. Produced cDNA was diluted 1:500 for the 18S assay and 1:100 for test assays.

Reaction batches were set as follows:

18S-standard		Sample	
Mastermix	12.5 µl	Mastermix	12.5 µl
dH_2O	12.5 µl	dH_2O	1.75 µl
Primer probe mix	1.25 µl	Primer 1 (100 µM)	0.25 µl
		Primer 2 (100 µM	0.25 µl
		Probe (10 µM)	0.25 µl

10 µl of the cDNA dilutions were provided into the wells of a MicroAmp Optical 96-well Reaction Plate and 15 µl of the mastermix were added. The plate was closed and centrifuged shortly. Real-time PCR was carried out using the TaqMan Cycler 7900HT ABI Prism (Applied Biosystems, Darmstadt, D) under the following conditions.

50 ° C	2 min
95 °C	10 min
50 cycles with:	
95 °C	15 sec
60 °C	1 min

Evaluation of data was performed with Sequence Detection Software SDS2.2

2.2.27 Orthotopic mouse model

To evaluate the influence of Col V *in vivo*, an orthotopic mouse model was used, where PSC with a knock-down of Col V together with SU.86.86 were implanted. Eight week old athymic (BALB/c nu/nu, Charles River) mice were grouped randomly into 4 groups with 5 mice each. 500 000 SU.86.86 cells were mixed with 500 000 stably transfected PSC and resuspended in 50 µl Matrigel™. To detect the formation of metastatic sites, tumor cells had been tagged with lacZ using lentiviral transduction. Before surgery an analgetic (Temgesic®) was applied intraperitoneally, then mice were anesthetized and an incision was made in the left flank. The spleen and the tail of the pancreas were exteriorized

and cells diluted in Matrigel™ were injected into the tail of the pancreas. Six weeks after surgery, mice were sacrificed, the pancreas removed, tumor size was measured and the tumors were weighed. Tumors were further processed for histologic examination and X-gal staining was done for 18 hrs to detect the presence of metastases. Surgery of the mice was done in cooperation with Dr. Tao Cheng, Department of Surgery, Klinikum rechts der Isar, München.

2.2.28 Statistical analysis

Results are expressed as mean ± standard error of the mean. Statistical analysis was performed using the software Spotfire S+ (TIBCO Software Inc, Palo Alto, CA, USA). Two-way analysis of variance was used to determine the significance. Graphs were generated using Microsoft Excel software. Correlation was calculated using GraphPad PRISM with the Pearson correlation.

3 Results

3.1 Expression of collagen type V in pancreatic cancer progression

Immunohistochemistry was performed with a Col V antibody on human tissue microarrays of precursor lesions (PanIN-1 to PanIN-3) and whole tissue slides of PDAC. Col V staining was seen in the stroma around PanIN lesions in a basement membrane-like pattern. In invasive carcinoma, the distribution of the staining was more diffuse and could also be observed within the cytoplasm of stromal cells (figure 5), whereas normal pancreas did not show any Col V expression (data not shown).

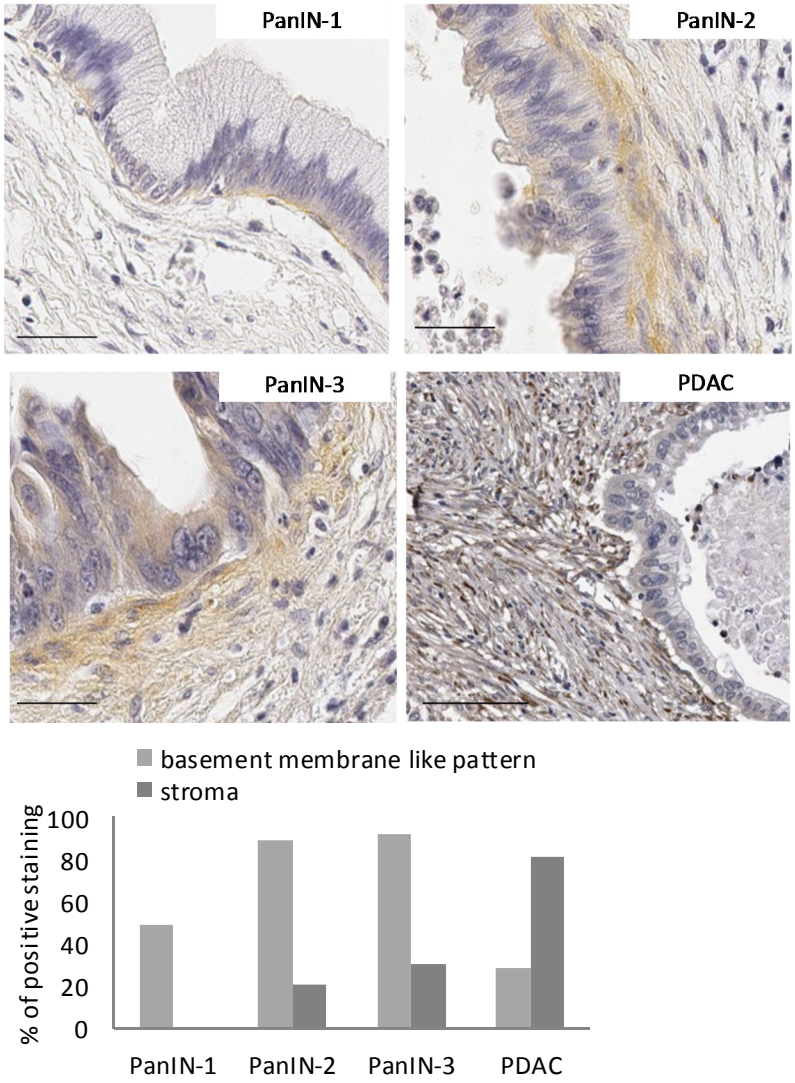

Figure 5: Immunohistochemical staining for Col V in human PanIN and PDAC. In the PanIN lesions (PanIN-1, 2 and 3) a basement membrane-like pattern is seen. This pattern disappears upon cancer progression and development of the desmoplastic stroma. Here, a more diffuse staining throughout the stromal compartment is observed, with an additional staining also seen within the cytoplasm of perilesional fibroblasts. A summary of the percentage of tissues stained positive in the basement membrane-like pattern and in the stroma during the cancer progression is shown in the graphical representation.

To address the possibility whether Col V is expressed by PSC, immunohistochemical co-staining for αSMA (a known marker of activated PSC) and Col V was performed on human pancreatic cancer tissues sections (Figure 6A). Here Col V was detectable in a fiber-like pattern within the stroma and a colocalization of Col V and αSMA was observed, indicating that PSC – at least in part – produce Col V. To validate this hypothesis, immunoblotting was performed on cell extracts from cultured human PSC obtained from PDAC patients, in four different cultured PCC (AsPC-1, Capan-1, PANC-1 and SU.86.86) and in HeLa cells. Immunoblotting confirmed the results obtained from the immunohistochemical double staining, showing a strong expression of Col V in PSC and a weak expression on one of the PCC (SU.86.86) and in the HeLa cells (Figure 6B).

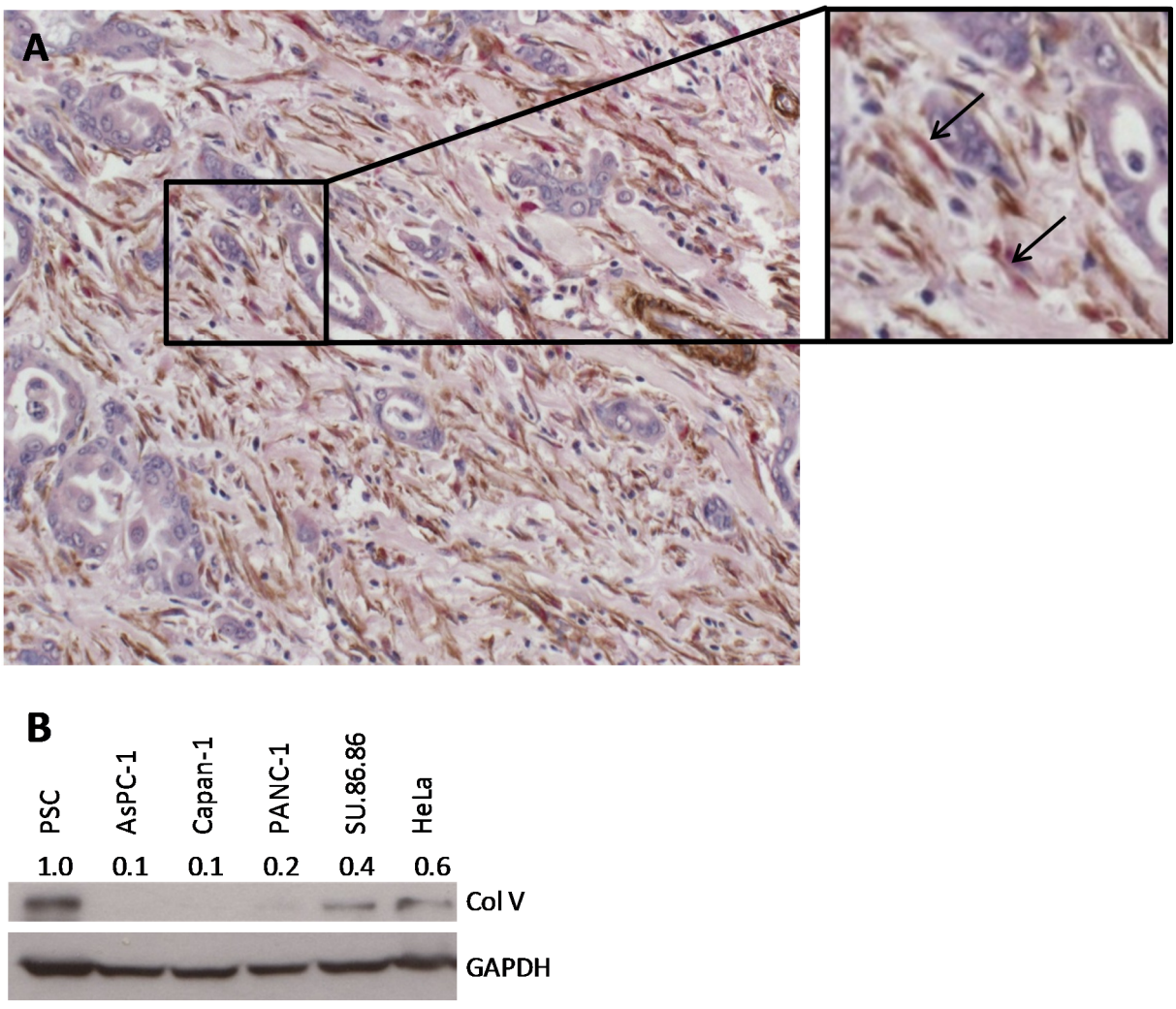

Figure 6: Expression of Col V in pancreatic cancer tissue, PSC and PCC. (A) Double staining in a human pancreatic cancer specimen with αSMA (brown) and Col V (red). Staining of Col V and αSMA is colocalized in the majority of the stromal cells, indicating PSC as the major source of Col V. (B) Immunoblotting of cell extracts from PSC, PCC and HeLa. A strong expression in the PSC could be seen, whereas only SU.86.86 cancer cells and the HeLa cells showed a weak expression. Numbers indicate the quantification of the expression.

Subcellular localization and expression of Col V was examined by double immunofluorescence on freshly isolated human PSC. As shown in figure 7, PSC in culture showed an abundant expression of αSMA, proving that these cells are activated myofibroblasts. Additionally, a moderate cytoplasmic expression of Col V was found in all cells, further confirming PSC as producers of this protein also in cell culture.

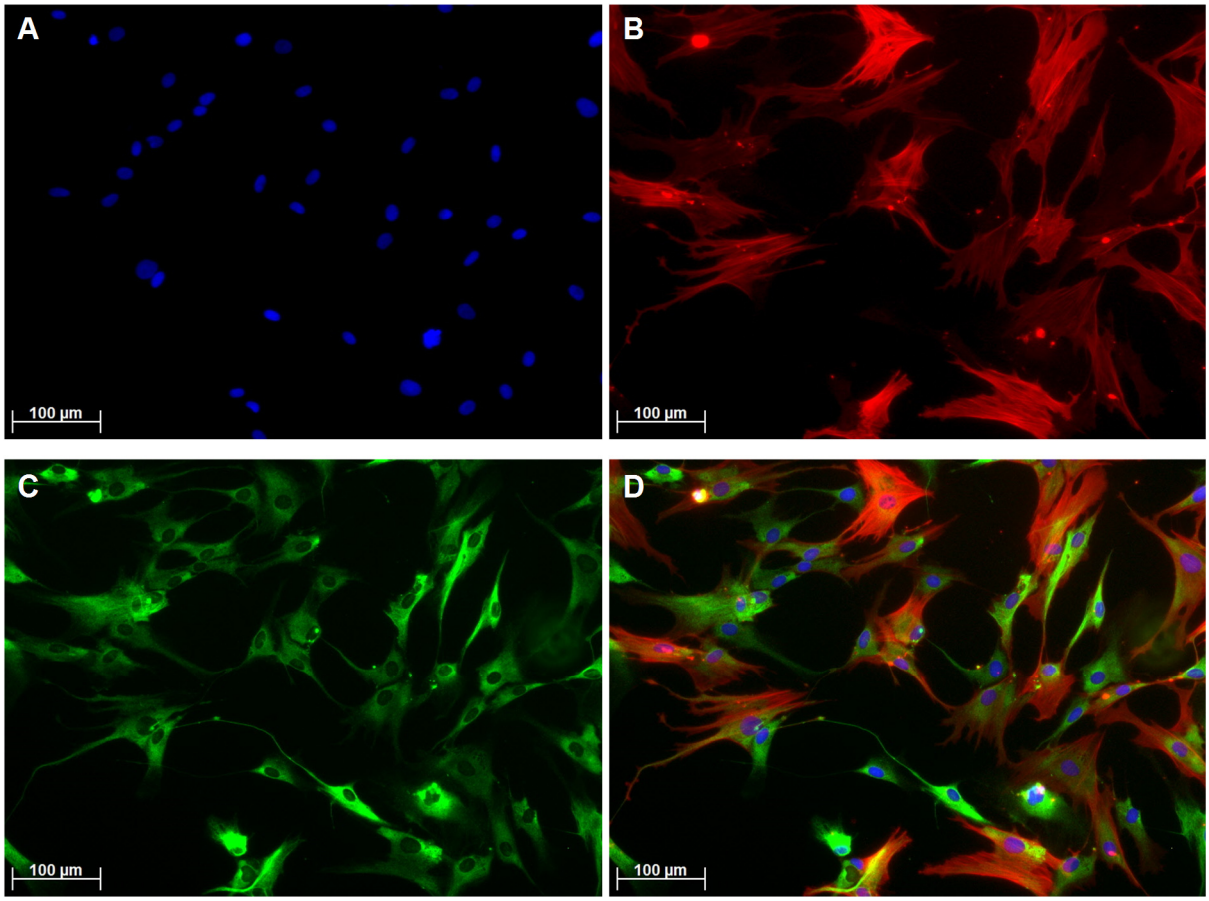

Figure 7: Immunofluorescence on PSC to determine the subcellular localization of Col V. The activation status was confirmed by αSMA (red) staining, Col V expression is shown in green. Cells were counterstained with Hoechst 33342. Expression of both proteins could be detected in all cells.

These results lead to the conclusion that PSC are the main producers of Col V in PDAC tissues.

3.2 Investigation of the effects of mutated Col V

In the previous sections it was shown that Col V in the pancreas is produced mainly by PSC. Another disease that is known to be affected by Col V is the classical Ehlers-Danlos syndrome (type 1 and 2). These patients bear different mutations in the Col V gene, which result in a connective tissue disorder, leading to highly elastic skin and joints. The skin fibroblasts of these patients have been already investigated and showed impaired ability of migration (Viglio, Zoppi et al. 2008). In the following experiments the properties of fibroblasts from Ehlers-Danlos patients were compared to normal skin

fibroblasts and PSC. First, it was tested by the means of immunofluorescence, if the Ehlers-Danlos fibroblasts (EDF) still express Col V. Figure 8 shows the staining of two different EDF cell lines (F011 and F057) stained for αSMA and Col V. Normal human dermal fibroblasts (HDF) were used as controls. EDF retained Col V expression at lower levels comparable with HDF and PSC, as seen in immunofluorescence. The expression level of Col V in these cell lines was further verified with immunoblotting (Figure 8). With this method, a slight difference in the Col V expression was found. The HDF expressed Col V in a higher amount than the EDF, but expression levels of PSC were comparable to the expression levels of EDF.

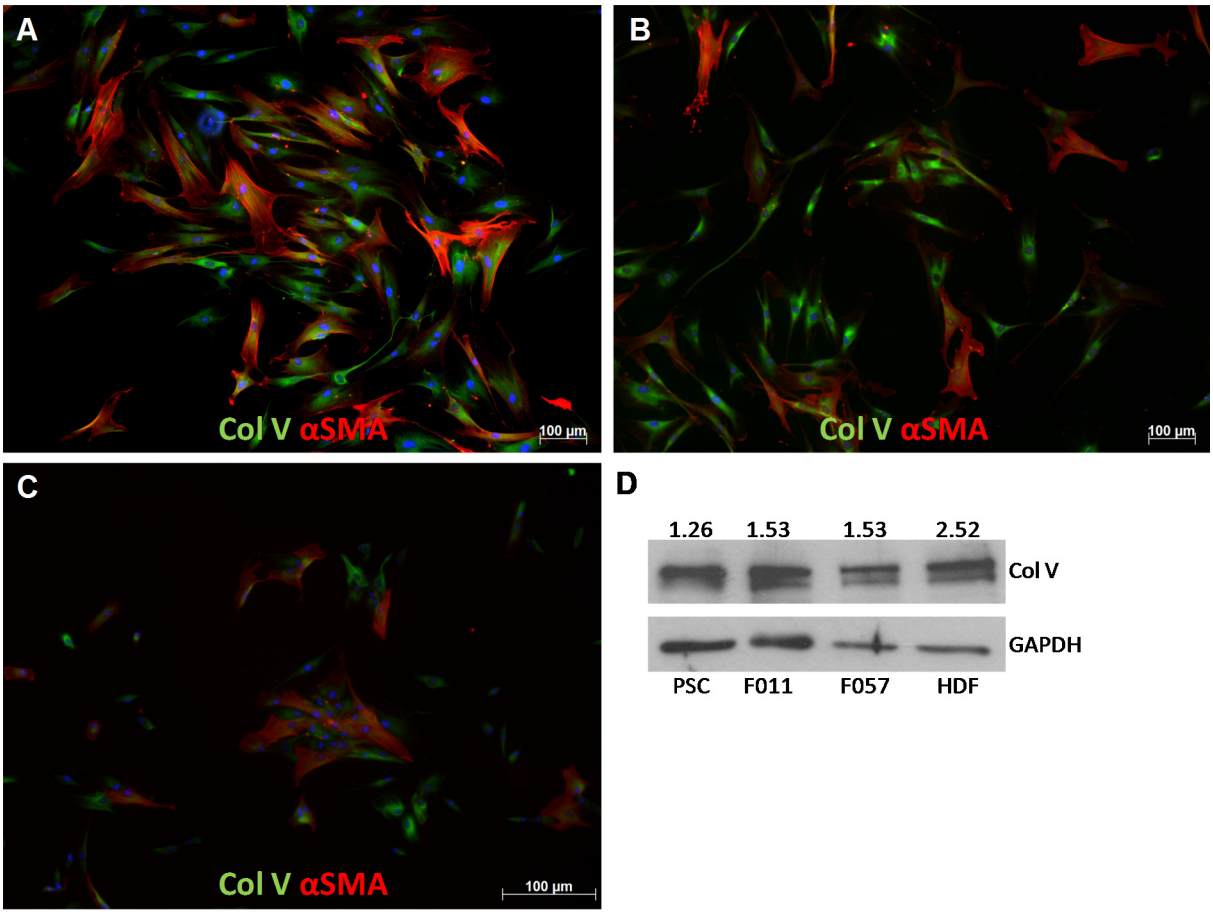

Figure 8: Immunofluorescence of EDF and HDF (A: F011, B: F057, C: HDF). A co-staining of αSMA and Col V is seen in all cell lines (A-C). Col V expression was verified in immunoblotting. EDF and PSC express similar amounts of Col V, whereas HDF express Col V in a higher content. Numbers indicate the quantification of the expression.

To investigate whether mutation of Col V affects functional properties of EDF, adhesion assays were performed and the results were compared with those obtained in PSC and HDF (Figure 9). Grown on control matrix, PSC showed a higher adhesion compared to mutated fibroblasts and HDF (F057: $p=0.039$, HDF: $p<0.01$). Same effects were observed when grown on a Col V-rich matrix ($p<0.001$). In

direct comparison of the adhesion on the control matrix to the Col V-rich matrix, all three types of fibroblasts showed a significant higher adhesion when grown on Col V ($p<0.001$).

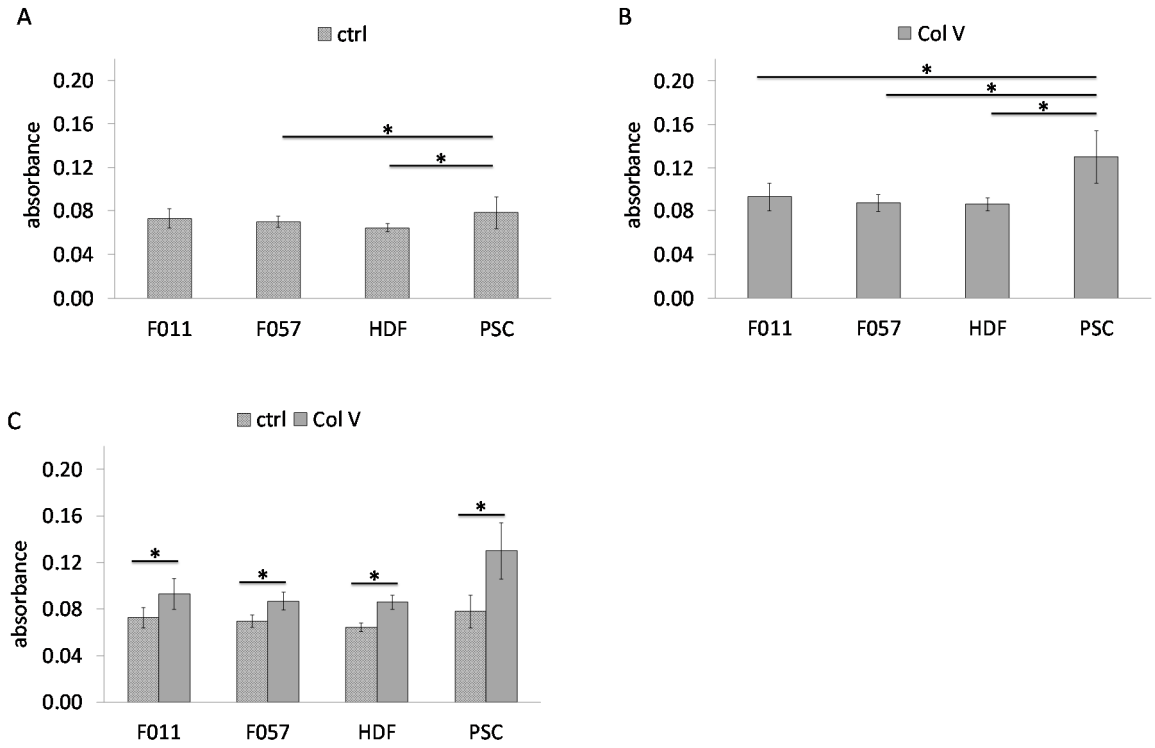

Figure 9: Adhesion of F011, F057, HDF and PSC. (A) Adhesion on the control matrix showed a significant increase for PSC compared to EDF and HDF (F057: $p=0.039$, HDF: $p<0.01$). (B) A significant increase in adhesion was seen on a Col V-rich matrix for PSC ($p<0.001$). (C) All types of fibroblasts showed a higher adhesion on the Col V-rich matrix compared to the control matrix ($p<0.001$).

MTT assay was used to test whether Col V can also influence the proliferation rates of these cells. Cells were seeded on a Col V-rich matrix and a control matrix and proliferation was measured after 48 hrs (Figure 10). A significantly stronger proliferation was found for HDF and PSC as compared to the EDF on control matrix as well as on Col V-rich matrix ($p<0.001$). In addition, the Col V-rich matrix increased proliferation of EDF and PSC (F011: $p=0.027$; F057: $p=0.005$; PSC: $p=0.036$), but not of HDF.

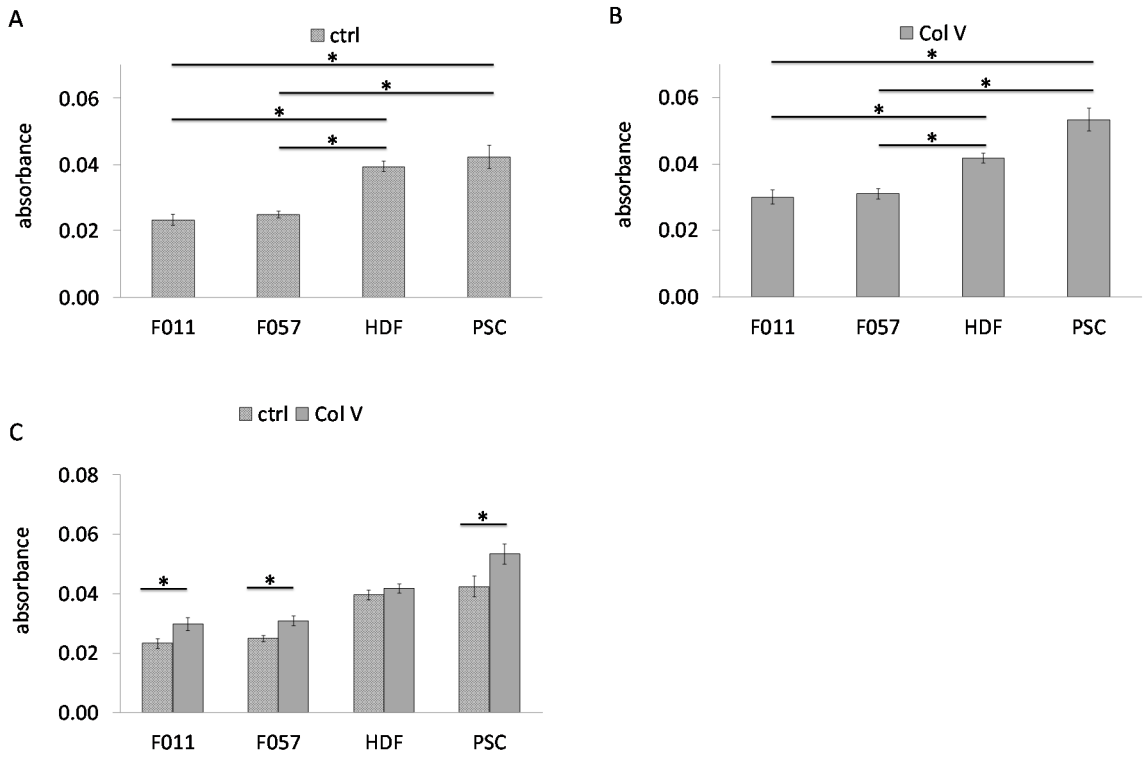

Figure 10: Proliferation assay using F011 and F057 in comparison to HDF and PSC. (A) Proliferation was significantly increased for HDF and PSC compared to EDF on the control matrix ($p<0.001$). (B) A significant increase in proliferation was also seen on the Col V-rich matrix for PSC and HDF ($p<0.001$). (C) EDF and PSC showed a significantly higher proliferation on Col V-rich matrix as compared to the control matrix (F011: $p=0.027$; F057: $p=0.005$; PSC: $p=0.036$).

Col V influences wound healing, as seen for example in patients affected by the classical Ehlers-Danlos syndrome (type 1 and 2) (Malfait, Wenstrup et al. 2010). Continuative cell migration was assessed by performing wound healing assays. Cells were seeded again on Col V coated plates and the migration was followed for 48 hrs (Figure 11). On the control matrix a significant higher migration was found for HDF and PSC ($p<0.001$) compared to the EDF. On the Col V-rich matrix, a significantly higher migration was found for HDF (F011: $p=0.028$; F057: $p=0.017$) and PSC ($p<0.001$). As expected, migration of the EDF was enhanced significantly by Col V (F011: $p<0.01$; F057: $p=0.046$) and also for PSC ($p=0.022$).

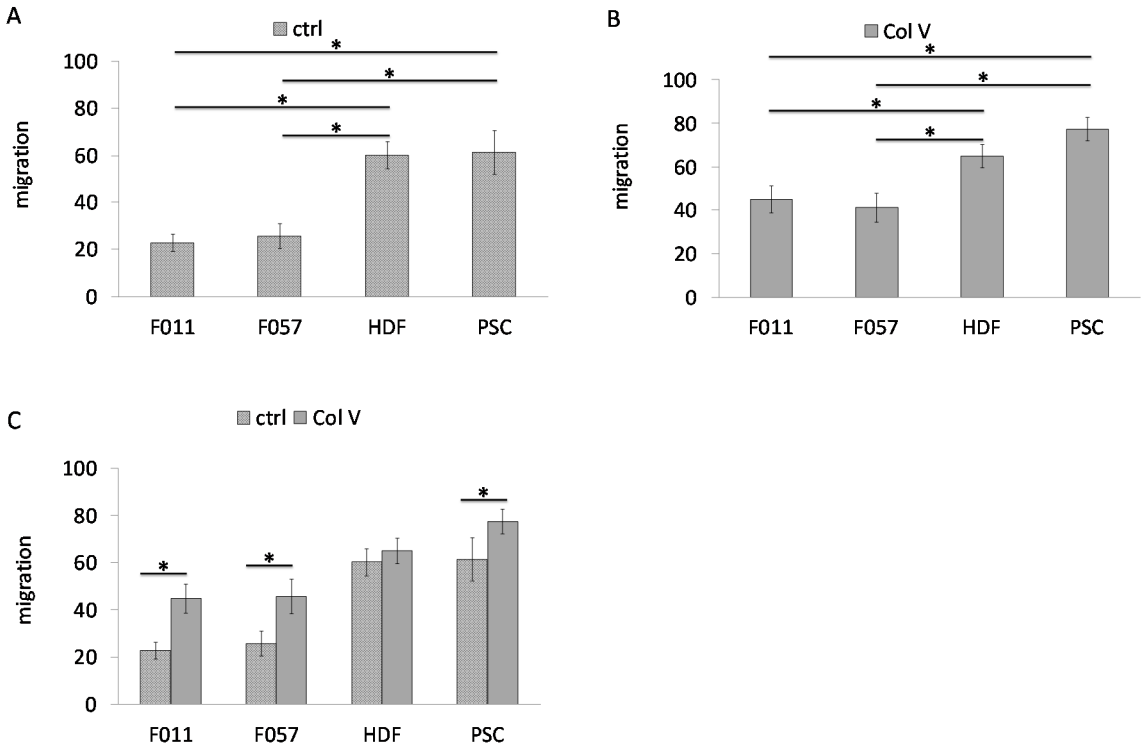

Figure 11: Migration assay using F011 and F057 in comparison to the HDF and PSC. (A) Migration was significantly increased for HDF and PSC compared to EDF on control matrix ($p<0.001$). (B) A significant increase in migration was seen also on the Col V-rich matrix for PSC ($p<0.001$) and HDF (F011: $p=0.028$; F057: $p=0.017$). (C) EDF and PSC showed a significantly higher migration on the Col V-rich matrix compared to the control matrix (F011: $p<0.01$; F057: $p=0.046$; PSC: $p=0.022$).

In summary, these data show that intact Col V is important for the functional properties of stromal cells and that exogenous Col V can further promote these properties and shift stromal fibroblasts to a more deleterious phenotype.

3.3 Collagen type V-dependent signaling pathways

To examine the functional role of Col V in PDAC, Col V dependent signaling pathways were investigated. First, expression pattern of β_1-integrin was investigated in PanIN and PDAC by immunohistochemistry (Figure 12). In the PanIN lesions a change in the expression pattern correlating with the grade of dysplasia was observed. Low grade lesions displayed a preferentially basolateral expression, whereas high grade lesions showed an apical and cytoplasmic staining (Figure 12A). In tumor tissues a moderate to strong membranous expression as well as moderate intracytoplasmic staining was observed (Figure 12B). No correlation between expression pattern and grading was observed in PDAC.

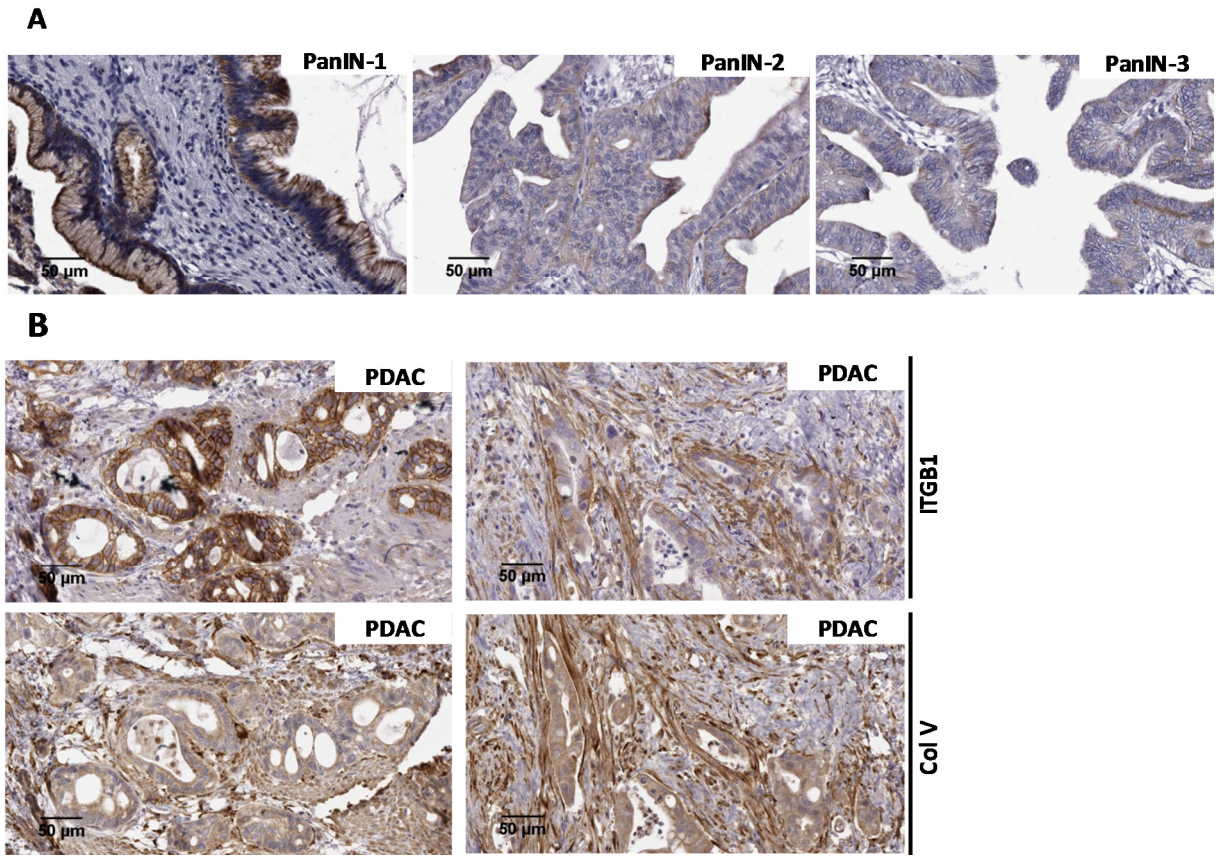

Figure 12: Immunohistochemistry of β_1-integrin (A, B) and Col V (B) in PanIN (A) and PDAC (B). The low grade PanIN shows a membranous basolateral expression with a slight cytoplasmic staining, whereas the staining in the high grade lesions is more disheveled, apical and cytoplasmic (A). The tumor tissues, with inter-tumoral variability, show a membranous or cytoplasmic staining in the cancer cells and partially in stromal cells. Col V expression is seen in the stroma surrounding the areas of β_1-integrin positive areas in consecutive sections (B).

In order to verify the expression of β_1-integrin in the PCC and PSC *in vitro*, immunoblotting was performed. Total protein was extracted from the cell lines and detected with a specific antibody against β_1-integrin (Figure 13A). In order to assess the effects on the β_1-integrin expression on a Col V-rich matrix on PCC, cell extracts were obtained from cells seeded on Col V as growing substrate. Bovine serum albumin (BSA) coated plates were used as control plates (Figure 13B). All PCC and the PSC showed expression of β_1-integrin on control plates, and a slightly increased expression was observed in the cell line SU.86.86 when grown on a Col V matrix. The cell lines AsPC-1 and Capan-1 did not show changes in their β_1-integrin expression when grown on Col V.

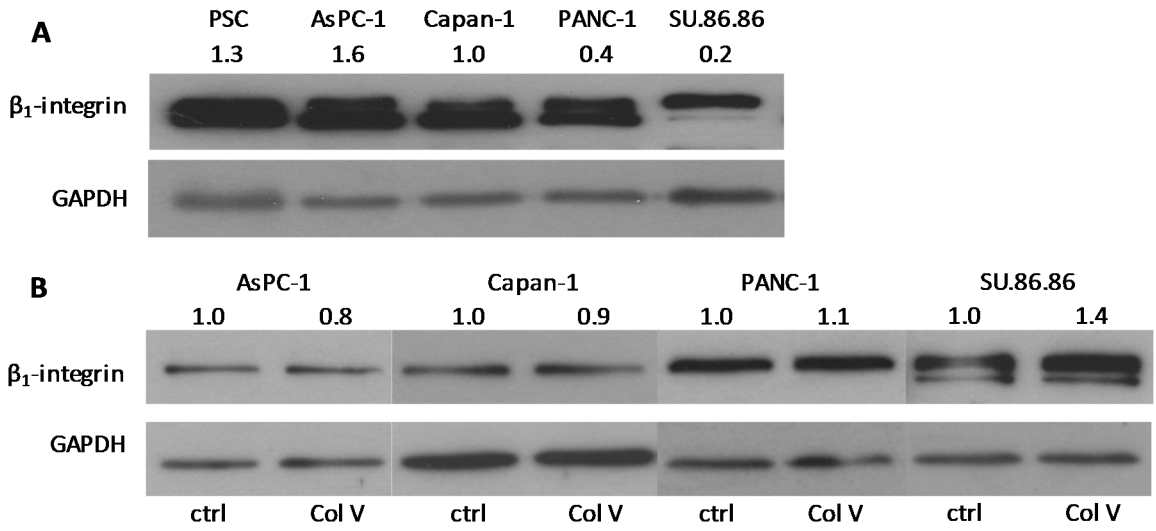

Figure 13: Expression of β_1-integrin in PCC and PSC. (A) All cell lines express β_1-integrin. (B) The cell line SU.86.86 shows a slight increase in the β_1-integrin expression grown on a Col V-rich matrix, but for the cell lines AsPC-1, Capan-1 and PANC-1 similar expression β_1-integrin was detected. Numbers indicate the quantification of the expression.

Downstream targets of the integrin signaling pathway are the kinases PAX and FAK. This pathway is known to influence the growth and migration of cancer cells (Larsen, Tremblay et al. 2003). To investigate whether this pathway is activated under the influence of Col V, immunoblotting was performed using antibodies against pFAK-Y397 and pPAX-Y118. GAPDH was used as loading control. To exclude that the upregulation is due to a higher expression of total FAK and PAX, the membranes were stripped and reprobed with antibodies against the unphosphorylated forms of FAK and PAX. For the cell lines AsPC-1, Capan-1 and SU.86.86 upregulation of pFAK-Y397and pPAX-Y118 could be detected 15 min after seeding for AsPC-1 and Capan-1 and 30 min after seeding for SU.86.86. No influence was seen for the cell line PANC-1 (Figure 14).

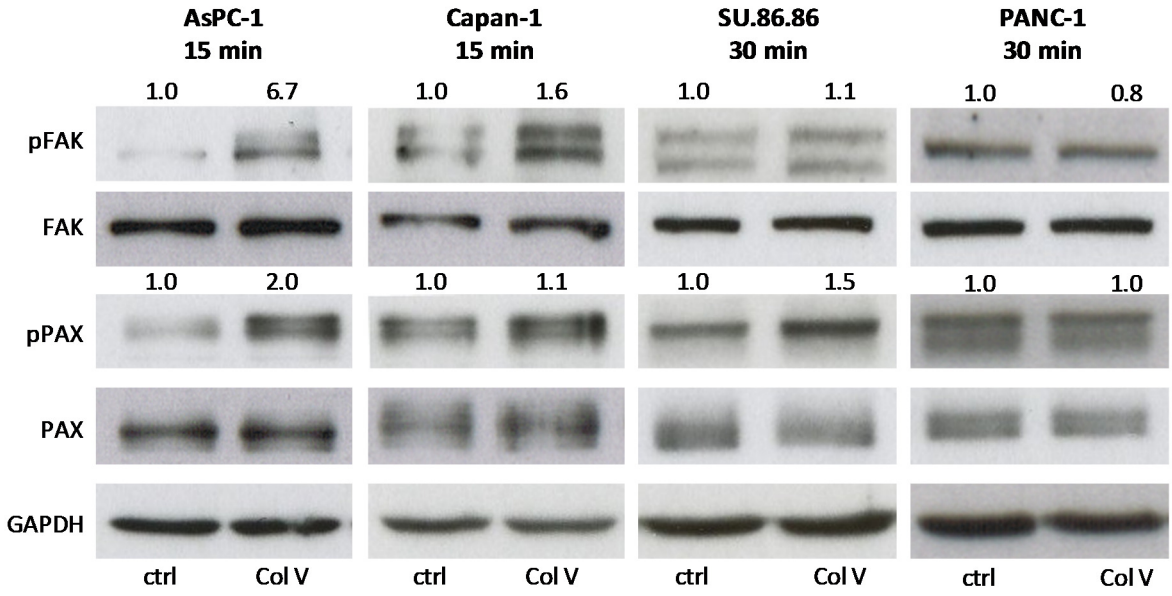

Figure 14: Integrin signaling pathway. Upregulation of pFAK-Y397 and pPAX-Y118 of PCC grown on a Col V-rich matrix could be detected in the cell lines AsPC-1, Capan-1 and SU.86.86. No upregulation could be shown when probing the unphosphorylated proteins, indicating the activation of the integrin signaling pathway. In PANC-1 no upregulation of pFAK-Y397 and pPAX-Y118 could be detected. Numbers indicate the quantification of the expression.

In a previous work (Berchtold 2009), the effects of the activation of this pathway on cancer cell morphology were investigated. BSA coated plates were used as control (ctrl) and pictures were taken after 3 hrs (Figure 15). While cells on the control plates after 3 hrs were just slightly attached to the surface, the cells on a Col V-rich matrix showed a drastic change in their morphology. They adhered strongly and displayed lamellipodia. In comparison, the cells grown on the control matrix were still round without migration sites and adherent to a lesser content. Col V therefore acts as a pro-adhesive substrate for PCC.

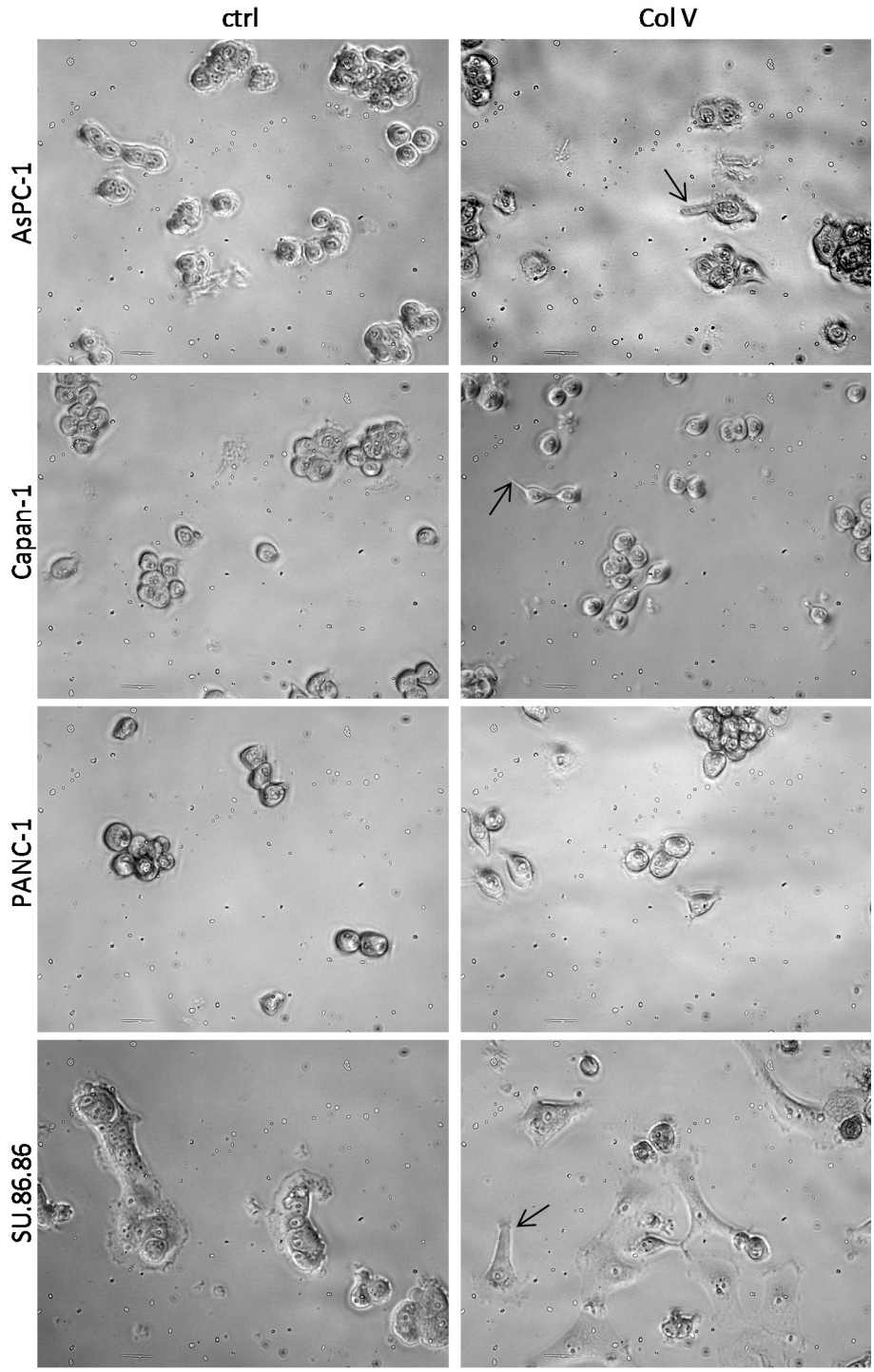

Figure 15: Morphology of different cancer cell lines on control plates and Col V coated plates. Pictures were taken 3 hrs after plating. The cells grown on a Col V-rich matrix show a more flat morphology compared to the cells grown on the control matrix, indicating faster adhesion onto the plates. Further, on the Col V-rich matrix the cells already start to build lamellipodia, which are important for the migratory properties of the cells.

With the technique of immunofluorescence labeling, the expression pattern and localization of the proteins pFAK-Y861 and pPAX-Y118 were examined. The cell line SU.86.86 was seeded on Col V-rich matrix or on a control matrix. Cells were stained with antibodies against pFAK-Y861, pPAX-Y118 and

the cytoskeletal protein vinculin (Figure 16). Investigation was done after 3 and 24 hrs after plating. After 3 hrs, a strong difference in the morphology of the cells was present according to the substrate. The cells on Col V were attached to the substrate and started to build up lamellipodia (migration sites). After 24 hrs, the cells grown on Col V strongly adhered to the surface and displayed increased numbers of lamellipodia, which are of great importance for cell migration. Staining for pFAK and pPAX was seen clearly at the migration edges (lamellipodia) of the cells, whereas the structural protein vinculin was evenly distributed throughout the whole cell and no difference between cells on control plates and Col V coated plates was observed.

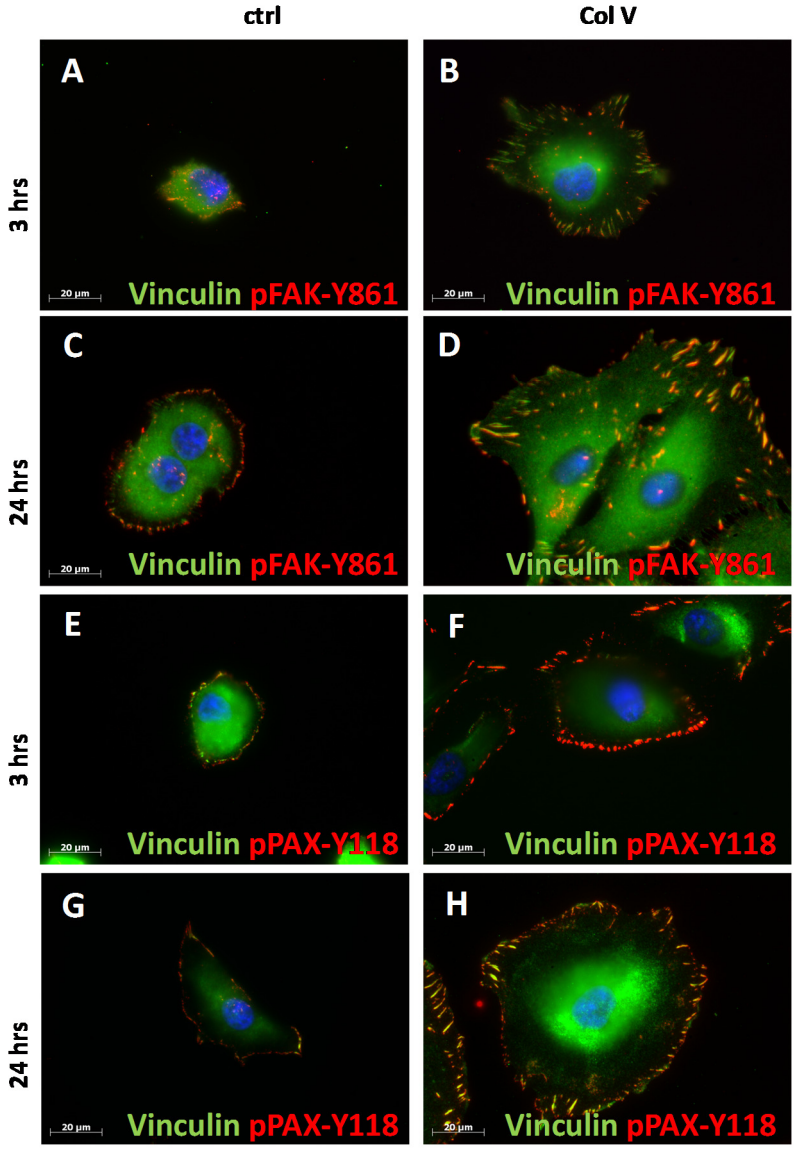

Figure 16: Immunofluorescence staining of pFAK-Y861 (A-D), pPAX-Y118 (E-H) and vinculin. Immunofluorescence staining was performed after 3 (A,B,E,F) and 24 hrs (C,G,D,H) either on control plates (A,C,E,G) or a Col V-rich matrix (B,D,F,H). A strong change in the morphology could be observed when the cells were grown on the Col V-rich matrix compared to the cells on the control matrix. Formation of lamellipodia was already present after 3 hrs on the Col V-rich matrix. The staining for pFAK-Y861 and pPAX-Y118 was concentrated at the migration sites.

Taken together, these results show that the β_1-integrin signaling pathway is involved in downstream signaling of Col V. PCC grown on Col V showed increased expression and different distribution of migration sites, suggesting a promoting effect of Col V on cell migration

3.4 Functional influence of collagen type V on pancreatic cancer lines

To further investigate the influence of Col V on PCC, different *in vitro* assays were performed using Col V as growing substrate for the cancer cells. The supposed pro-adhesive effect of Col V for PCC was evaluated using an adhesion assay (Berchtold 2009). The cells were seeded on a Col V-rich matrix and allowed to adhere for 3 hrs; after that cells that were still not attached were washed away and the adherent cells were fixed with methanol, stained with toluidine blue, lysed and the absorbance was measured using an ELISA-Reader (Figure 17). Confirmation of the previous findings concerning the morphology could be achieved with the adhesion assay. The cell lines AsPC-1, Capan-1 and SU.86.86 adhered significantly better on the Col V-coated surface. No significant change for the cell line PANC-1 was seen.

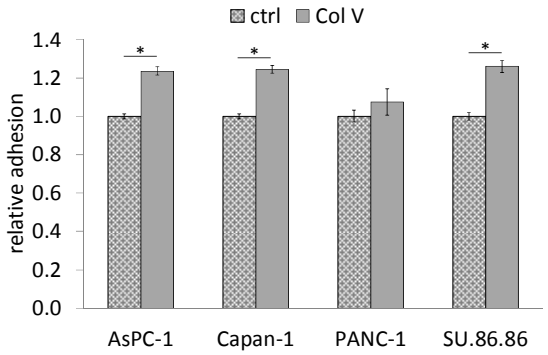

Figure 17: Effects of exogenous Col V on the adhesion of the cell lines AsPC-1, Capan-1, PANC-1 and SU.86.86 after 3 hrs. The cell lines AsPC-1, Capan-1 and SU.86.86 show a significant increased adhesion after 3 hrs on a Col V-rich matrix compared to the adhesion of the cells on the control plates (AsPC-1, SU.86.86: $p<0.01$; Capan-1: $p=0.035$).

The effects of Col V on cell viability and proliferation were assessed by a proliferation assay (MTT) (Figure 18). All cell lines (AsPC-1, Capan-1 and SU.86.86) that exhibited already a stronger adhesion on Col V also showed increased proliferation when grown on a Col V-rich matrix (AsPC-1, SU.86.86: $p<0.01$; Capan-1: $p=0.024$).

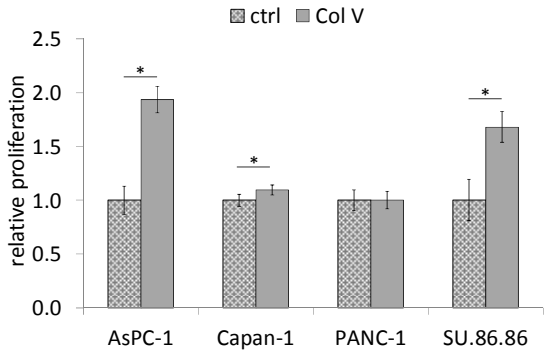

Figure 18: Effects of exogenous Col V on proliferation of the cell lines AsPC-1, Capan-1, PANC-1 and SU.86.86. Proliferation rates of cells grown on Col V for 24 hrs in standard culture medium were compared to those of cells grown on BSA coated control plates. A significant difference was seen in AsPC-1, Capan-1 and SU.86.86 (AsPC-1, SU.86.86: $p<0.001$, Capan-1: $p=0.024$). The proliferation of PANC-1 was not affected by Col V.

To examine whether Col V affects the migratory behavior, wound healing assays were performed. Cells were grown in a monolayer either on a control matrix or on a Col V-rich matrix. The cells were then starved for 24 hrs in serum free medium and the monolayer was scratched with a p10 pipette tip. Pictures of the open wound were taken after 24 and 48 hrs (Figure 19). Concordant to the effects on adhesion and proliferation, an increased migration could be determined (24 hrs: Capan-1 $p<0.01$, SU.86.86 $p<0.001$; 48 hrs: Capan-1 $p<0.05$, SU.86.86 $p<0.01$). AsPC-1 was excluded from this assay, as the cells did not form a monolayer.

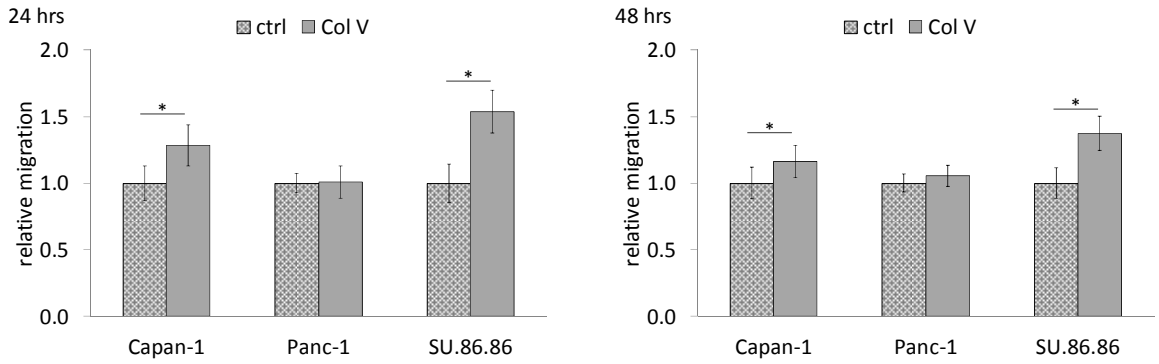

Figure 19: Effects of exogenous Col V on the migration of the cell lines Capan-1, PANC-1 and SU.86.86. The migration was followed for 48 hrs. Open area was measured and compared to the open area at 0 hrs. The cell lines Capan-1 and SU.86.86 showed a significant increase of the migration up to 48 hrs on Col V (24 hrs: Capan-1 $p<0.01$, SU.86.86 $p<0.001$; 48 hrs: Capan-1 $p<0.05$, SU.86.86 $p<0.01$).

Since the composition of the ECM is known to affect chemoresistance (Neesse, Michl et al. 2011), it was tested if Col V could also influence the survival of cancer cells upon treatment with 5-FU and Gem. For this purpose, cells were treated with 5-FU or Gem and viability was tested by MTT after 72 hrs. As anticipated, the administration of 5-FU induced a decrease in the viability of the cancer cells. Still under

the influence of 5-FU, a higher proliferation rate could be detected when the cells were grown on a Col V-rich matrix. This effect was significant for the cell lines Capan-1 and SU.86.86 ($p<0.05$) (Figure 20, upper row). Additionally to the experiment with 5-FU, similar results were obtained with the administration of Gem (Figure 20, lower row). Here the cell lines AsPC-1, Capan-1 and SU.86.86 showed a higher proliferation rate on Col V, which was significant (AsPC-1: $p<0.05$, Capan-1: $p<0.001$ and SU.86.86: $p<0.05$) compared to the control plates.

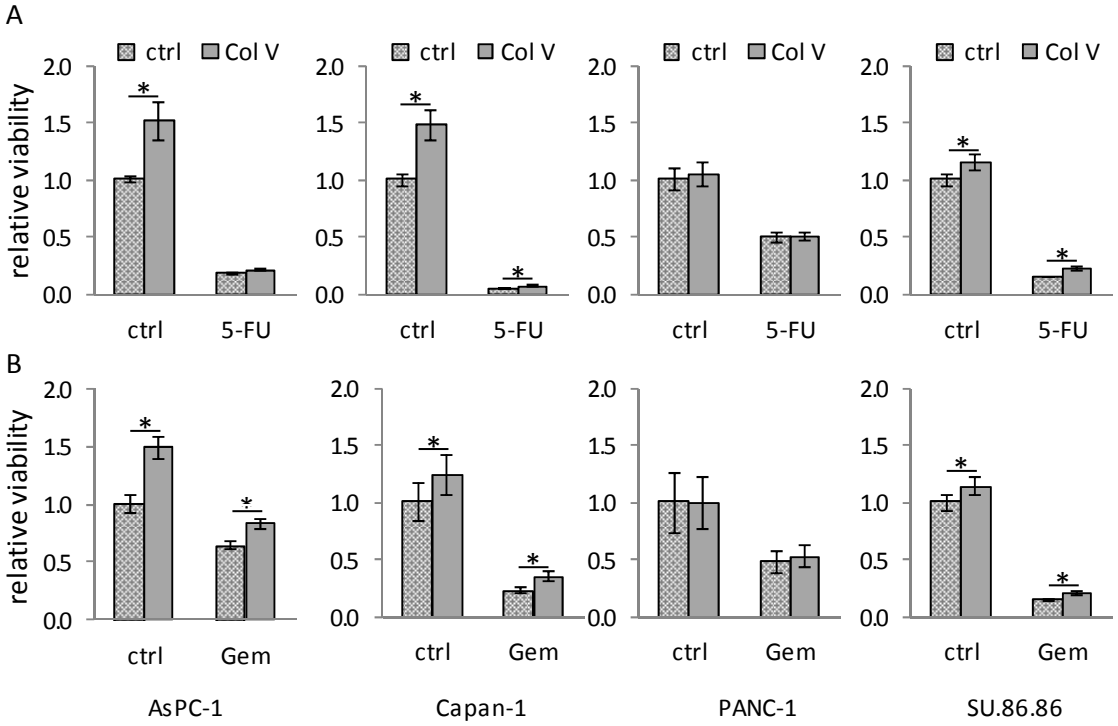

Figure 20: Proliferation of AsPC-1, Capan-1, PANC-1 and SU.86.86 grown on a Col V-rich matrix and treated with 1 mM 5-FU or 10 µM Gem for 72 hrs. (A) After treatment of the cells with the chemotherapeutic 5-FU a decrease in proliferation could be observed in all cell lines. Nevertheless, the cell lines AsPC-1, Capan-1 and SU.86.86 showed an increased proliferation on Col V compared to the control plates, which was significant for Capan-1 ($p<0.05$) and SU.86.86 ($p<0.05$). (B) Cells grown on the Col V-rich matrix showed a higher proliferation rate compared to the cells on the control plates also after treatment with Gem. This effect was significant for the cell lines AsPC-1 ($p<0.05$), Capan-1 ($p<0.001$) and SU.86.86 ($p<0.05$).

In order to further elucidate the mechanisms involved in the improved survival of cancer cells under the influence of Col V, apoptosis assays and cell cycle analysis were performed using the cell line SU.86.86. Cells were seeded on a Col V-rich matrix and treated with 10 µM Gem and 1 mM 5-FU, respectively. The analysis of apoptotic cells was done after 0 hrs, 24 hrs, 48 hrs and 72 hrs (Figure 21). Neither the treatment with Gem, nor with 5-FU resulted in different rates of apoptosis.

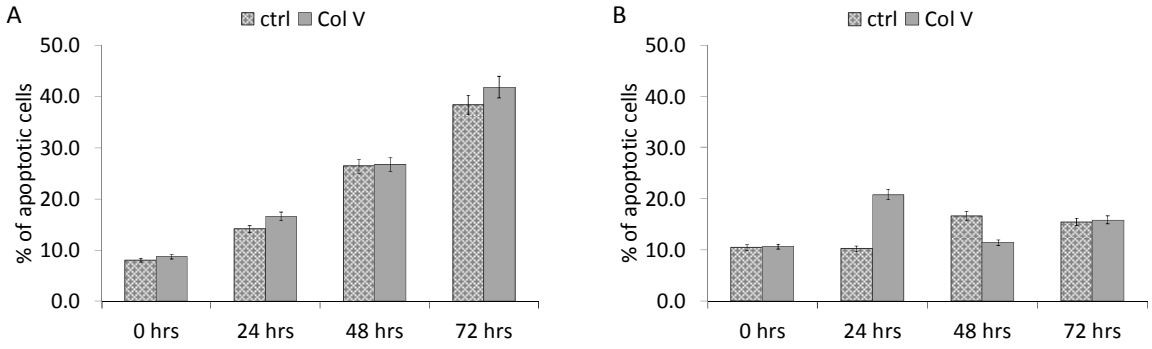

Figure 21: FACS analysis of apoptotic cells. SU.86.86 cells were treated with 5-FU (A) and Gem (B) respectively. Neither in the cells treated with Gem nor the cells treated with 5-FU a significant difference between cells grown on a Col V-rich matrix and cells grown o the control matrix could be seen.

For cell cycle analysis, SU.86.86 cells were starved for 96 hrs to allow cell cycle synchronization, before standard culture medium was added to start the cell cycle again (Figure 22). Cells grown on Col V showed a higher proliferation rate compared to the cells on the control plates. The same effect was evident when the cells were treated with 10 µM Gem for up to 72 hrs. This result confirms the previous results shown in the MTT. Col V has a proliferation stimulating effect that still exists under the influence of drug treatment.

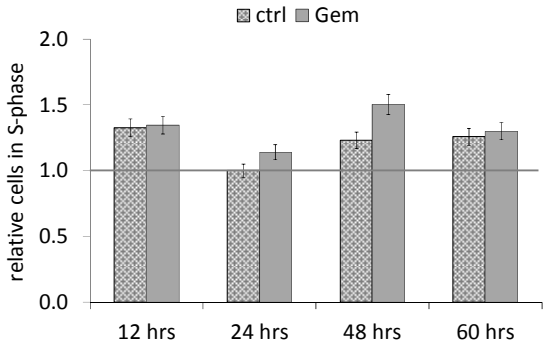

Figure 22: Cell cycle analysis of the cell line SU.86.86 either untreated or treated with Gem. Cell cycle synchronization was achieved with serum deprivation for 96 hrs, before normal medium was added together with or without Gem. The cell cycle was investigated up to 72 hrs after administration of Gem. A higher proliferation rate on Col V was seen in both conditions either with or without Gem. Results of S-phase were normalized to S-phases of cells grown on control plates. Flow cytometry analysis was done once.

Taken together, the results concerning adhesion, proliferation and migration show that exogenous Col V affects the malignant phenotype of PCC *in vitro*. Moreover, Col V promotes the survival of cancer cells under the influence of two different chemotherapeutics.

3.5 Inhibition of the integrin signaling pathway

To verify whether the integrin signaling pathway is related to the observed effects of Col V on cancer cells, an inhibition of the downstream molecules was conducted. First, the small molecule inhibitor AZD0530 (AZD) was used. AZD targets Src kinase and blocks the downstream signaling pathway including the kinases FAK and PAX. The kinase domain pFAK-Y567/577 was investigated, since Src-dependent phosphorylation occurs at this domain. Effective downregulation of the β_1-integrin signaling pathway was achieved with the inhibitor. Upon inhibition with AZD an almost complete inhibition of pFAK-Y576/577 and pPAX-Y118 could be detected in cells grown on a standard matrix and a Col V-rich matrix (Figure 23).

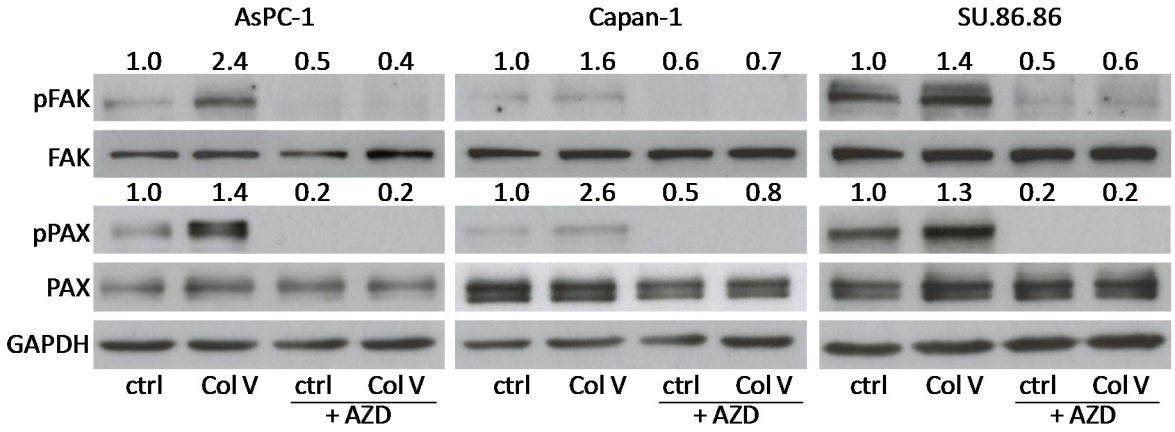

Figure 23: Integrin signaling pathway after treatment with the Src-inhibitor. AsPC-1, Capan-1 and SU.86.86 were seeded either with or without AZD on a Col V-rich matrix or a control matrix. Protein extraction was done after 15 min for AsPC-1 and Capan-1 and after 30 min for SU.86.86. An inhibition of pFAK-Y576/577 and pPAX-Y118 could be detected for all cell lines. Numbers indicate the quantification of the expression. Phosphoproteins were normalized against unphosphorylated proteins and GAPDH.

Verification of these results was achieved with a blocking antibody against β_1-integrin, which specifically prevents the binding of Col V to its receptor. This provides a more specific inhibition of the activation through Col V. In this experiment the autophosphorylation site of FAK (pFAK-Y397) and phosphorylation pPAX-Y118 was investigated. A decreased expression of pFAK-Y397 and pPAX-Y118 could be detected in the tested cell lines on control plates and less intense on the Col V-rich matrix (Figure 24).

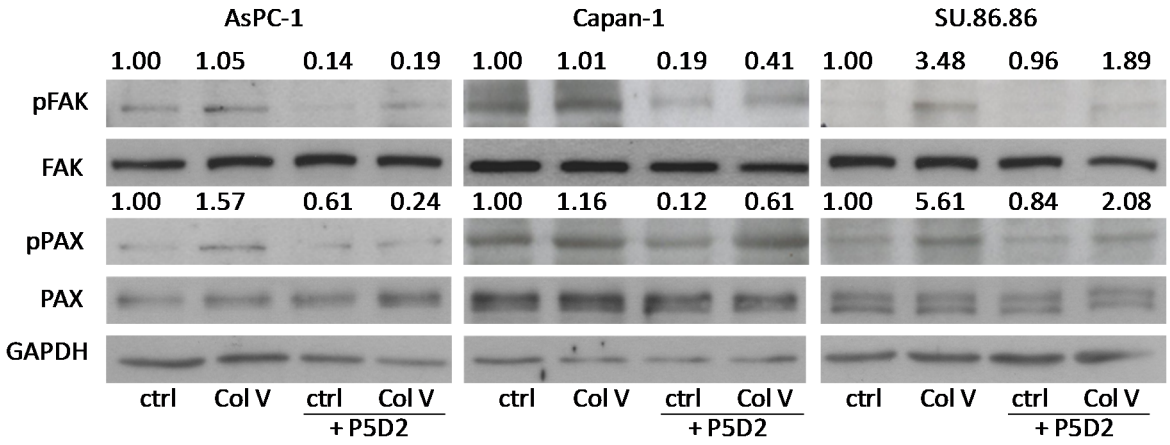

Figure 24: Integrin signaling pathway after treatment with the blocking antibody P5D2. AsPC-1, Capan-1 and SU.86.86 were seeded either with or without the blocking antibody P5D2, after initial incubation for 1 hr at standard conditions, on a Col V-rich matrix plates or control matrix. Protein extraction was done after 15 min for AsPC-1 and Capan-1 and after 30 min for SU.86.86. Detection of the proteins was conducted *via* immunoblotting. Phosphorylation of pFAK-Y397 and pPAX-Y118 was inhibited in all cell lines. Numbers indicate the quantification of the expression. Phosphoproteins were normalized against unphosphorylated proteins and GAPDH.

To determine if the inhibition of the β_1-integrin signaling pathway influences cellular behavior, the cells were seeded on Col V-rich matrix and treated with 2 µM AZD or the blocking antibody P5D2 (2.5 µg/1x10^6 cells). First, the adhesion of the cancer cells on Col V was determined (Figure 25). As described above, all the cell lines showed a significant increase in adhesion when grown on Col V compared to their controls. After AZD treatment (upper row) a significantly decreased adhesion was observed when cells were grown on Col V (AsPC-1: $p<0.001$, Capan-1: $p<0.05$, SU.86.86 $p<0.01$). Under the influence of AZD SU.86.86 cells showed a significantly higher adhesion when grown on Col V compared to the controls ($p<0.001$). Using the blocking antibody (lower row) a significantly decreased adhesion was observed in AsPC-1 ($p=0.046$) and Capan-1 ($p<0.001$) grown on Col V. Nevertheless cells still displayed better adhesion if grown on Col V (AsPC-1: $p=0.034$, Capan-1: $p<0.05$, SU.86.86 $p<0.01$).

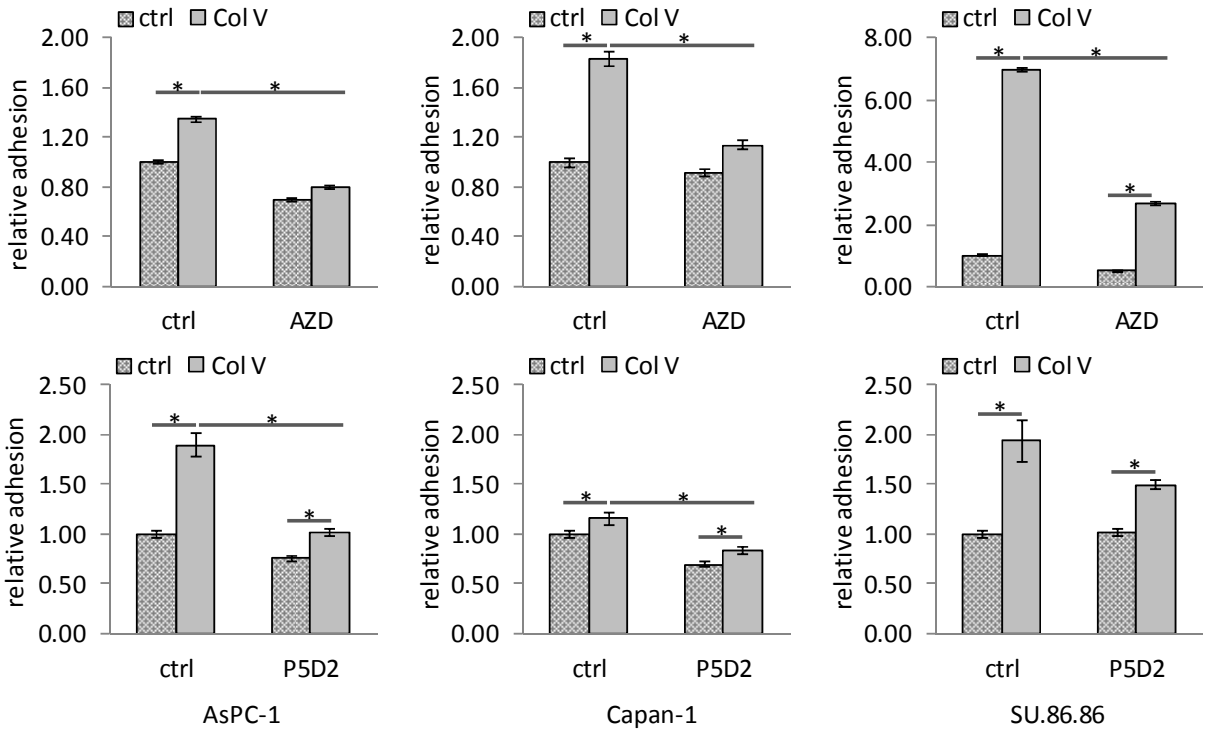

Figure 25: Adhesion of the cell lines AsPC-1, Capan-1 and SU.86.86 under the influence of the inhibitors AZD and P5D2. The adhesion was measured after 11 hrs for AsPC-1, after 19 hrs for Capan-1 and after 3 hrs for SU.86.86. Incubation with AZD inhibited the adhesion on Col V significantly compared to the untreated cells (AsPC-1: $p<0.001$, Capan-1: $p<0.05$, SU.86.86: $p<0.01$). Upon treatment with P5D2, adhesion was significantly inhibited for AsPC-1 ($p=0.046$) and Capan-1 ($p<0.001$). Cells seeded on Col V still adhered better than on control plates which was significant for SU.86.86 ($p<0.001$) for AZD and for all cell lines (AsPC-1: $p=0.034$, Capan-1: $p<0.05$, SU.86.86 $p<0.01$) for P5D2.

Previous results showed that Col V also influences the proliferation of the cancer cell lines. Therefore proliferation assays were performed using the inhibitors AZD and P5D2. The proliferation was measured after 24 and 48 hrs (Figure 26). Treatment with the inhibitors decreased proliferation for all cell lines, which was significant for AsPC-1 and Capan-1. Despite treatment, the proliferation of all cells lines was significantly increased when cells were seeded on Col V compared to the control.

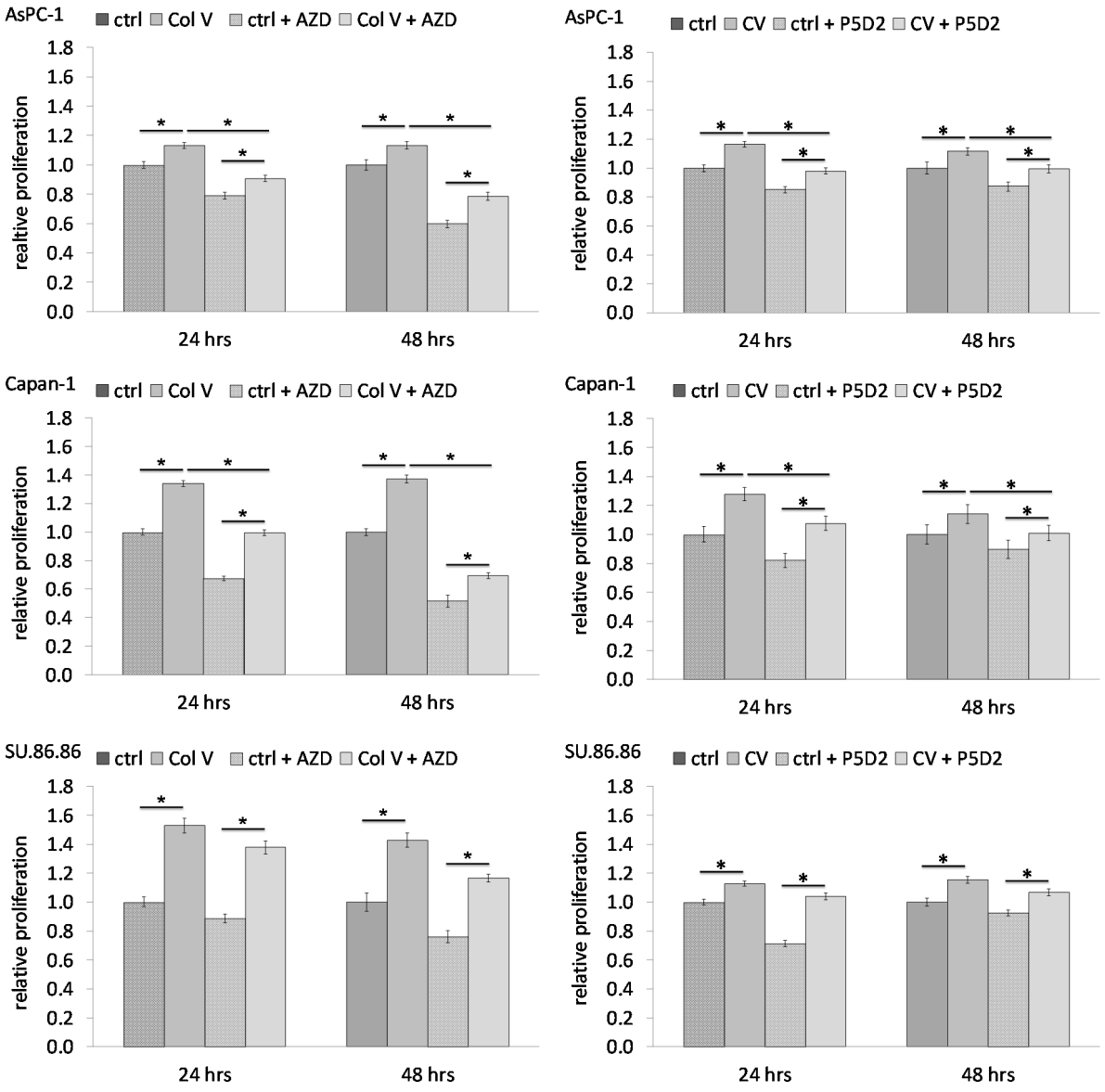

Figure 26: Proliferation of the cell lines AsPC-1, Capan-1 and SU.86.86 under the influence of the inhibitors AZD and P5D2. Proliferation was measured 24 and 48 hrs after administration of the inhibitors. The proliferation was reduced when cells were treated with AZD at 24 (AsPC-1: $p<0.01$ and Capan-1: $p=0.042$) and 48 hrs (AsPC-1: $p<0.001$ and Capan-1: $p<0.001$) and with P5D2 at 24 (AsPC-1: $p<0.01$ and Capan-1: $p=0.049$) and 48 hrs (AsPC-1: $p<0.01$ and Capan-1: $p=0.025$). A significantly increased proliferation under the influence of AZD after 24 (AsPC-1: $p=0.043$, Capan-1: $p=0.045$ and SU.86.86: $p=0.040$) and 48 hrs (AsPC-1: $p=0.032$, Capan-1: $p<0.01$ and SU.86.86: $p=0.045$) was achieved when the cells were grown on Col V. The same holds true for P5D2 after 24 (AsPC-1: $p=0.044$, Capan-1: $p=0.034$ and SU.86.86: $p=0.028$) and 48 hrs (AsPC-1: $p=0.018$, Capan-1: $p=0.047$ and SU.86.86: $p=0.032$).

To test the influence of the inhibitors on Col V-induced cancer cell migration, wound healing assays were performed. The closing of the wound was followed for 48 hrs. The cell line AsPC-1 was excluded in this analysis, because the cells do not form a monolayer, which is necessary for the evaluation. In both cell lines tested there was a significant increase of migration between control matrix and

Col V-rich matrix. After treatment with AZD and P5D2, migration on Col V-rich matrix decreased significantly already at 24 hrs, and the effect could still be seen after 48 hrs. Col V partly rescued the migration of SU.86.86 in both treatment groups (Figure 27).

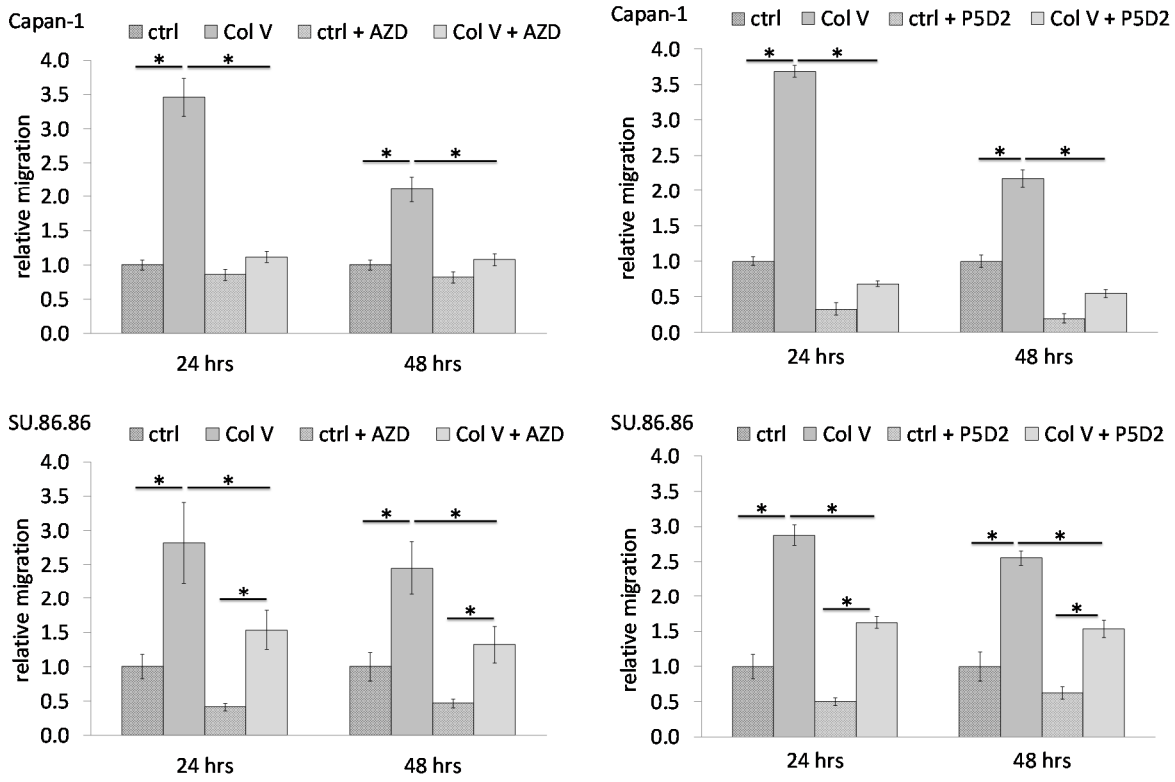

Figure 27: Migration assay under the influence of Col V and the inhibitor AZD. Results are shown for cell lines Capan-1 (A) and SU.86.86 (B). The migration was followed for 48 hrs. A significant decrease in the migration could be observed after 24 (Capan-1: p=0.035 and SU.86.86: p=0.049) and 48 hrs (Capan-1: p=0.029 and SU.86.86: p=0.018 and) after treatment with AZD and after 24 (Capan-1: p=0.049 and SU.86.86: p<0.01) and 48 hrs (Capan-1: p=0.024 and SU.86.86: p<0.01) after treatment with P5D2. SU.86.86 grown on Col V still migrate more than cells grown on control plates both after 24 (AZD: p=0.029 and P5D2: p<0.01) and 48 hrs (AZD: p=0.020 and P5D2: p<0.01).

Pancreatic cancer cells grown on Col V showed increased survival when treated with the chemotherapeutics 5-FU and Gem. Influence of the inhibitors on chemoresistance was investigated using a viability assay. While the cells treated with the chemotherapeutics showed a significantly higher viability when grown on Col V, the cells treated with the inhibitors showed a decrease in the viability, which is comparable to the cells grown on the control plates. Only the cell line SU.86.86 showed still a higher viability on Col V. Nevertheless, also here a significant decrease was observed between the treated and untreated cells (Figure 28).

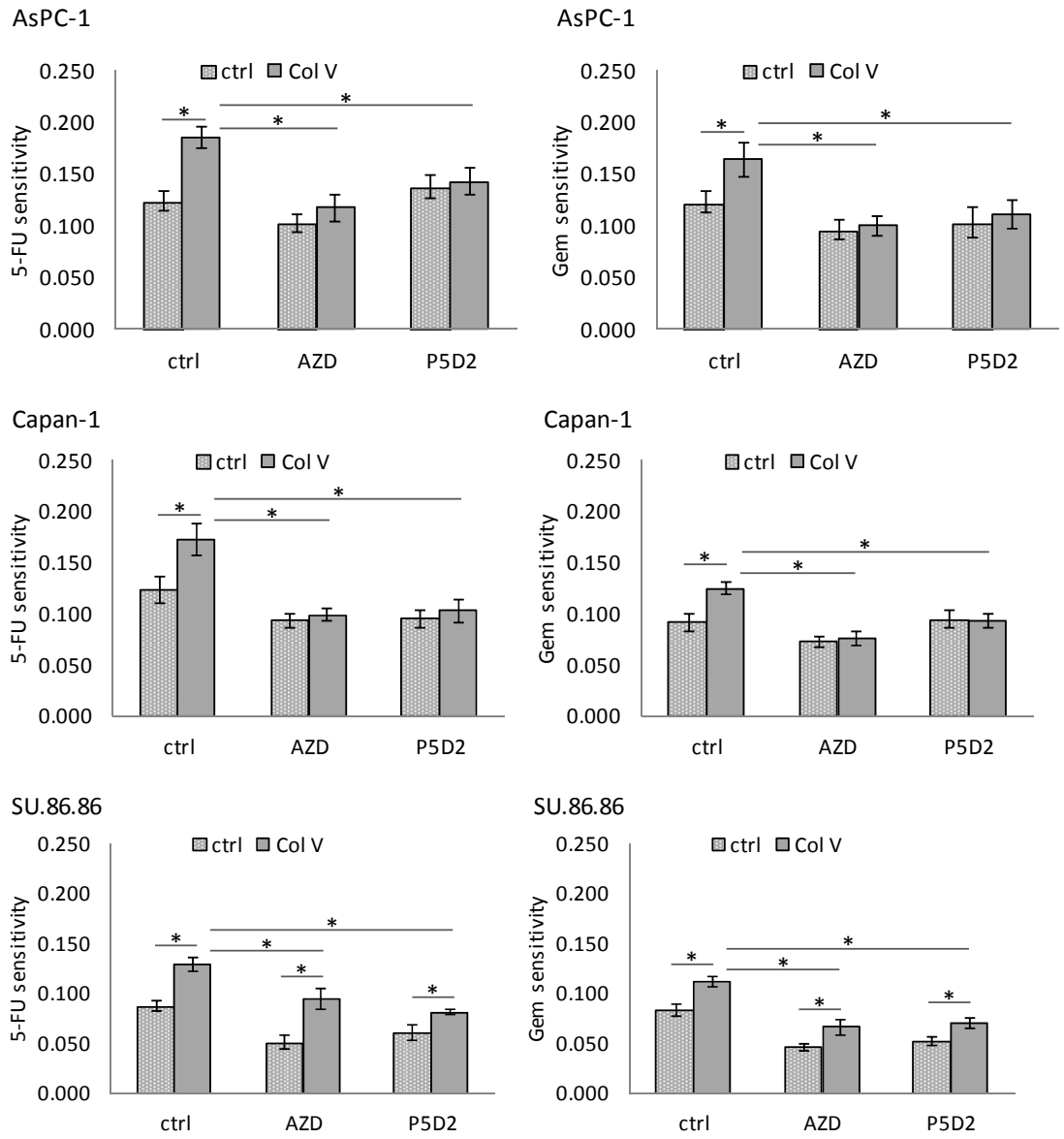

Figure 28: Chemoresistance of pancreatic cancer cell lines grown on Col V and treated with AZD and P5D2. Chemoresistance against 5-FU and Gem was reversed by AZD (AsPC-1: $p<0.001$, Capan-1: $p<0.001$ and SU.86.86: $p<0.05$) and P5D2 (AsPC-1: $p=0.026$, Capan-1: $p=0.034$ and SU.86.86: $p<0.001$) as well as by treatment with Gem by AZD (AsPC-1: $p<0.01$, Capan-1: $p<0.001$ and SU.86.86: $p<0.001$) and P5D2 (AsPC-1: $p=0.023$, Capan-1: $p<0.01$ and SU.86.86: $p<0.001$).

Altogether these experiments show that Col V-induced effects on cancer cell adhesion, proliferation, migration and chemoresistance are at least partially mediated by the β_1-integrin pathway.

3.6 Interaction between stellate cells and cancer cells

Since PSC are the main source of Col V in pancreatic cancer tissues, in the next set of experiments we focused on the effects of PSC on PCC. A knock-down of Col V was achieved *via* transient siRNA transfection, which was stable for at least one week. This is a quite long effect, making it possible to

perform several *in vitro* assays. CM obtained from siRNA treated PSC were collected and used for *in vitro* studies. In figure 29 the efficiency of the Col V knock-down with siRNA is shown.

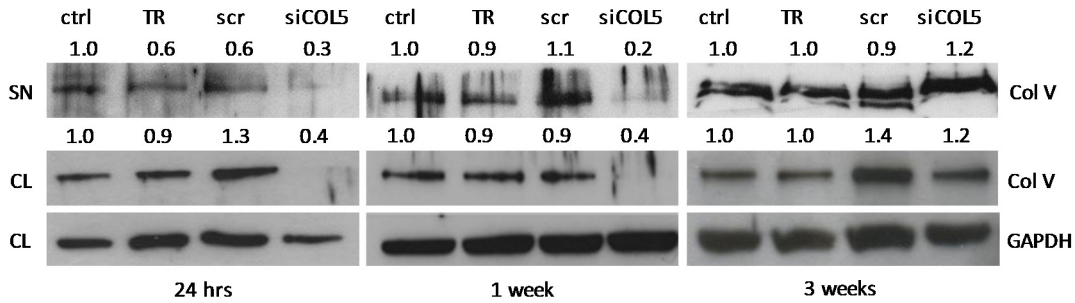

Figure 29: Western blot analysis to investigate the efficiency of transient Col V knock-down by siRNA. Proteins were extracted from control cells (ctrl), cells treated with transfection reagent (TR), cells treated with scrambled siRNA (scr) and cells treated with siRNA against COL5A1 (siCOL5). Protein extraction was performed after 24 hrs, one week and three weeks post transfection. The knock-down of Col V was stable for up to one week. After three weeks Col V expression was restored. Numbers indicate the quantification of the expression.

To address if the knock-down of Col V has an influence on the morphology and activation status of PSC, immunofluorescence was performed. Cells were stained with antibodies against Col V and αSMA (Figure 30). A change in the morphology was observed in the siRNA treated cells. The PSC lacking Col V showed an altered morphology. They appeared thinner, resembling more the morphology of normal fibroblasts, than activated myofibroblasts

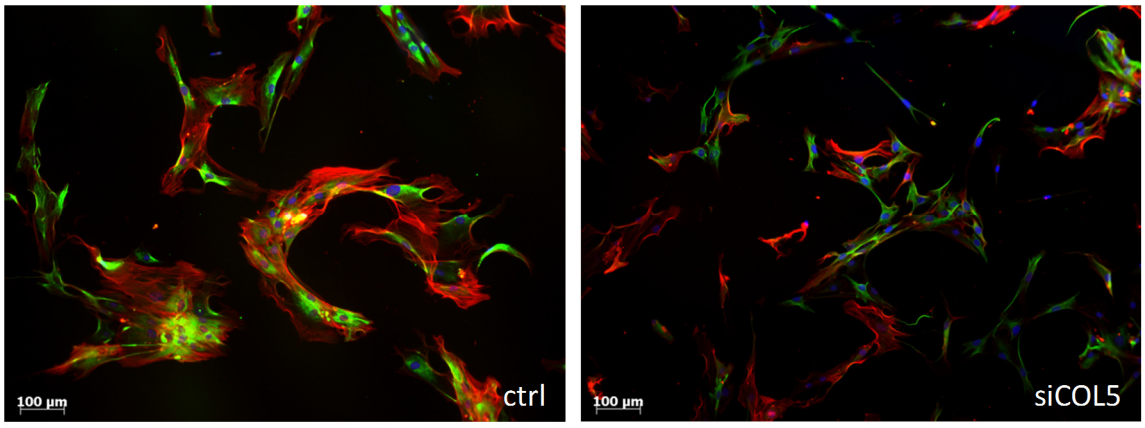

Figure 30: Immunofluorescence staining for Col V (green) and αSMA (red) before and after knock-down of Col V in PSC. Col V expression is decreased after siRNA treatment, whereas the expression of αSMA seems to be unaltered. The morphology of the treated cells is changed to a more fibroblast-like appearance.

To verify, if this change in morphology was related to a loss of the PSC phenotype, immunoblotting was done to quantify the expression of αSMA. The knock-down of Col V only changed the

morphological appearance but not the activation status, as no change in αSMA expression could be observed (Figure 31).

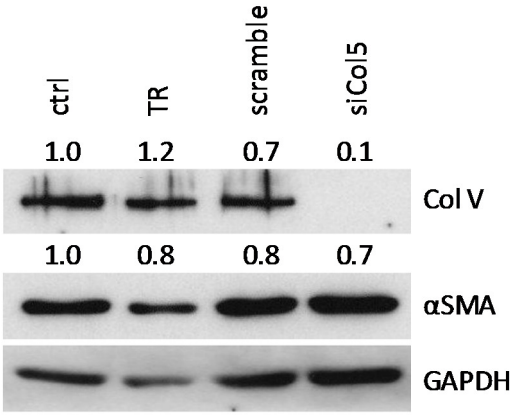

Figure 31: Immunoblotting of cell extracts from siRNA treated PSC. The knock-down of Col V is clearly visible, whereas no change in the expression of αSMA is observed. Numbers indicate the quantification of the expression.

Invasion is an important mechanism for cancer cells to build metastases. To investigate if secreted Col V in the interaction of PSC and PCC also has an effect on the invasiveness of the cancer cells, an invasion assay was performed. Therefore cancer cells were seeded on transwell chambers and as chemoattractant CM from untreated PSC or CM from siCOL5 treated PSC was used. Figure 32A shows the relative invasion between the cells treated either with the knock-down medium or the control medium. A change in the invasiveness and proliferation was detected when CM of siRNA treated PSC was used. The cell line Capan-1 showed a significantly ($p=0.035$) reduced invasion towards Col V-deprived PSC medium compared to the control medium of untreated PSC, while AsPC-1 and SU.86.86 were not significantly influenced. Regarding the proliferation (figure 32 B), a significant decrease was detected for the cell line AsPC-1 after 24 hrs ($p=0.049$), whereas no effects were seen in Capan-1 and SU.86.86 cells.

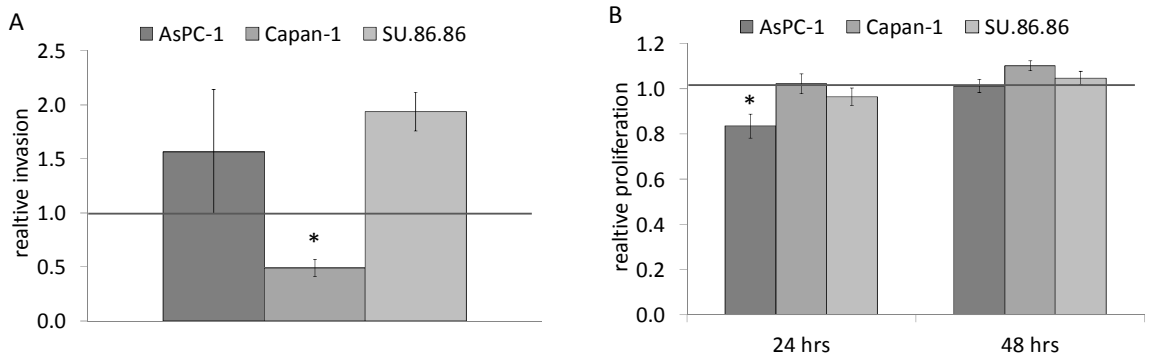

Figure 32: Effects of CM of siRNA-treated PSC against PCC. CM was obtained from PSC treated with siRNA against Col V and used as chemoattractant. (A) The invasive behavior of PCC against CM from PSC lacking Col V was measured using invasion assays. Capan-1 cells showed significantly reduced invasion when CM from siRNA treated PSC was used (p=0.035). (B) Proliferation of AsPC-1, Capan-1 and SU.86.86 treated with standard CM and CM from PSC treated with siRNA. A significant decrease of the proliferation with CM of siRNA treated PSC was seen only after 24 hrs for the cell line AsPC-1 (p=0.049).

To check the effects of PCC secretions on PSC with special emphasis on their Col V secretion, CM was obtained from PCC and PSC were incubated with it. 96 hrs later the supernatant was collected and protein extraction from cell lysates was performed (Figure 33). In the cell lysates no differences between the treated and the control cells could be noticed, but in the supernatants a higher amount of Col V could be detected with a fold change of 2.18 for AsPC-1, 1.73 for Capan-1 and 1.05 for SU.86.86 compared to the PSC grown in their standard medium, indicating that PCC especially influence Col V secretion from PSC.

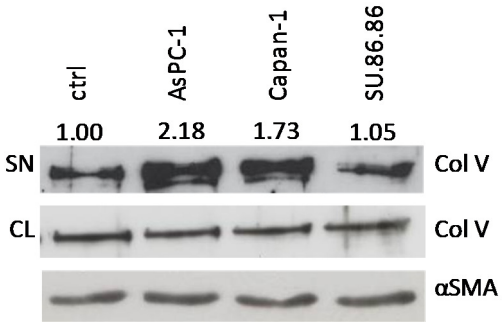

Figure 33: Secretion and expression of Col V in PSC after treatment with CM of PCC. PSC were treated for 96 hrs with the CM of cancer cells, before the Col V content in the supernatant (SN) and the expression in the cell lysates (CL) was investigated. In the cell lysates Col V expression was not altered, but a higher secretion of Col V was detected. Numbers indicate the quantification of the expression.

Taken together, these results show a decrease in proliferation and invasion in Col V-depleted conditions was found in PCC. Additionally, PCC-derived factors could increase the secretion of Col V in PSC.

3.7 Collagen type V and angiogenesis

Angiogenesis is fundamental in wound healing and Col V affects both processes. To assess the role of Col V in angiogenesis in pancreatic cancer, serial immunohistochemical stainings for Col V and CD34, a marker for human endothelial cells, were performed in forty resected human pancreatic tissue specimens (Figure 34). In figure 34, stromal expression in areas with neoangiogenesis, highlighted by CD34 immunostaining, is shown. No correlation between Col V expression and neoangiogenesis was found at the morphological level.

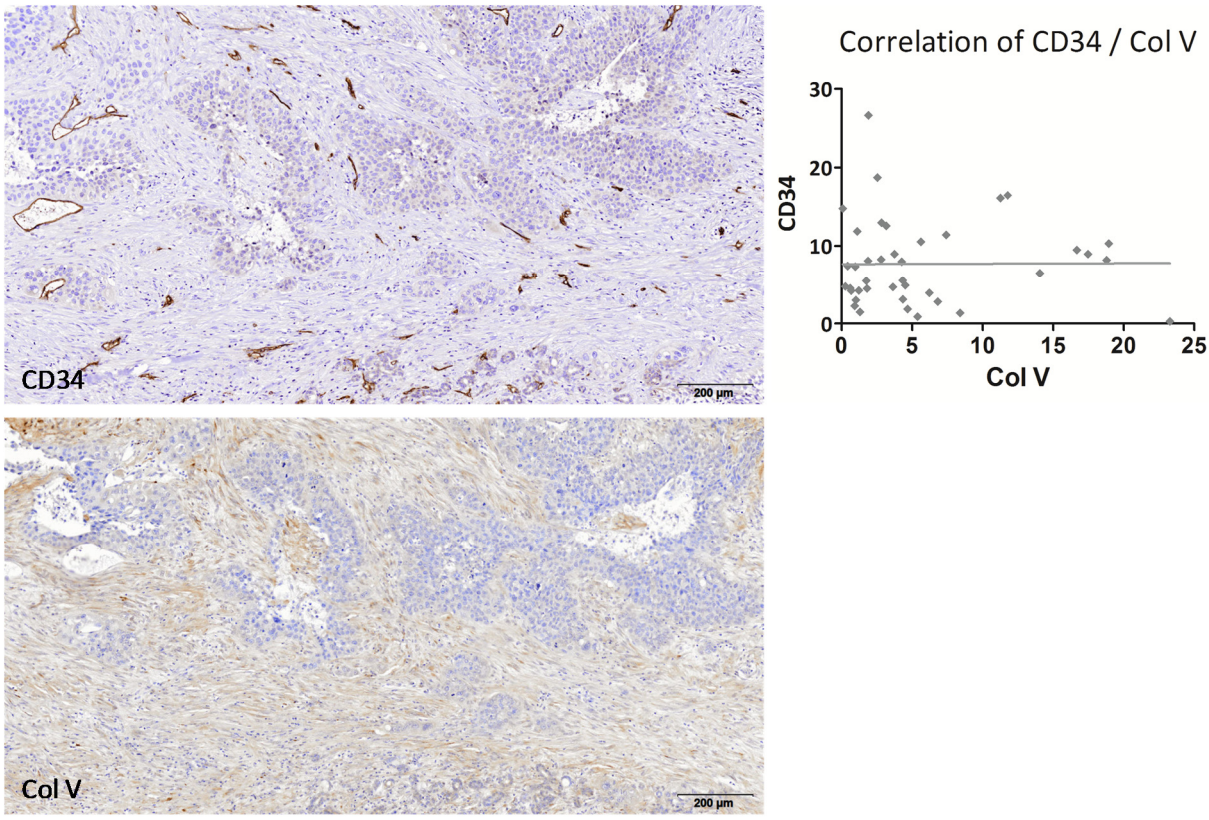

Figure 34: Representative immunohistochemical staining of CD34 and Col V. The upper picture shows the staining of CD34, a marker for endothelial cells. The same area was stained for Col V in the lower picture. The graph shows the summary of the semi-quantitative correlation of 40 investigated cases. No correlation between the two stainings could be observed (r^2=0.0029).

To further address the possible effects of Col V on neovascularization, tube formation assays with HUVEC, a human umbilical vein endothelial cell line, were performed using a Col V matrix compared to cells grown on control plates. A Col I matrix was used as positive control, as its effect on tube formation is already described (Montanez, Casaroli-Marano et al. 2002). The cells without collagen as substrate do not show any tube formation. HUVEC seeded on Col V and Col I show beginning tube formation, which was slightly more pronounced on Col I coated plates (Figure 35).

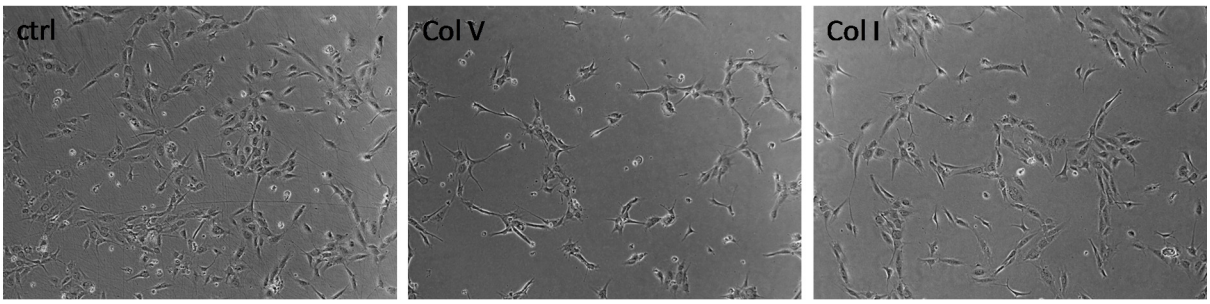

Figure 35: Tube formation assay with different coatings of cell culture plates. No tube formation could be detected when HUVEC cells were plated on normal culture plates. Using Col V and Col I as growth substrate a slight enhancement in tube formation on Col V could be seen. This phenomenon was slightly stronger on Col I.

To further investigate the role of Col V in the formation of tubes, Col V was knocked-down in HUVEC and tube formation assays with Matrigel™ were performed. Cells were seeded on Matrigel™ and tube formation was photographed after overnight incubation. The knock-down of Col V in HUVEC led to an impaired ability of these to form tubes. The controls with the untreated cells and the scrambled control showed normal tube formation (Figure 36).

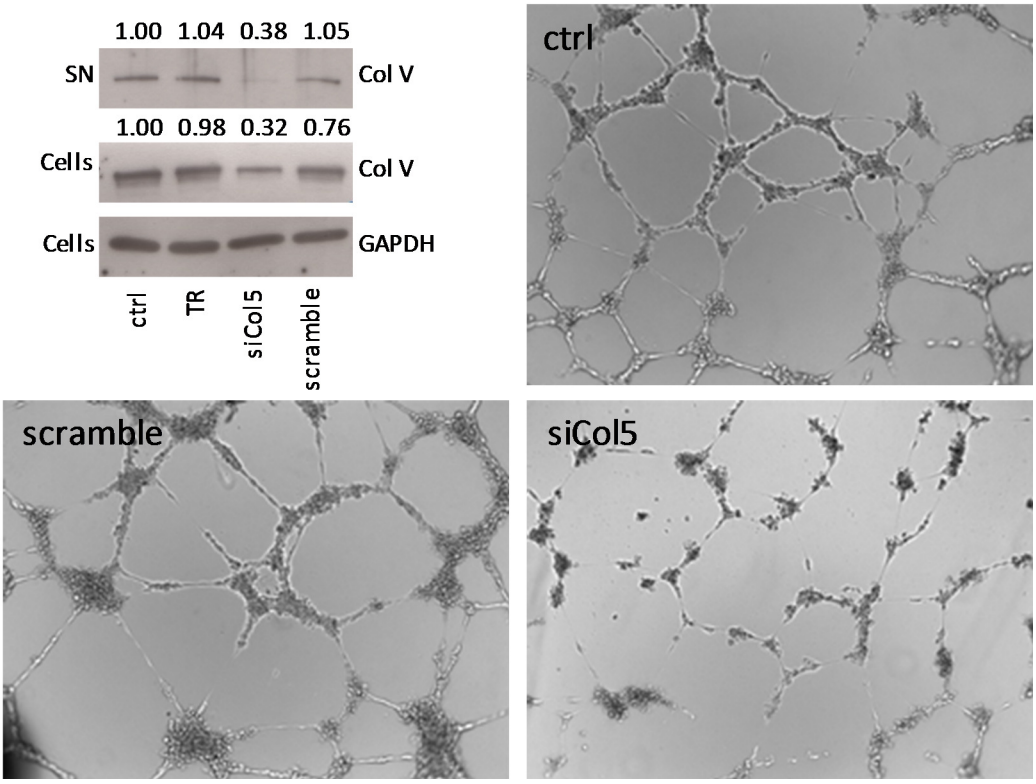

Figure 36: Tube formation assay with knock-down of Col V in HUVEC. Efficacy of siRNA-mediated knock-down in HUVEC was verified with immunoblotting. Tube formation assay with knock-down of Col V in HUVEC (siCol5), treated with scrambled siRNA (scramble) and untreated (ctrl) was performed. The untreated cells and the cells treated with scrambled siRNA showed normal tube formation. Knock-down of Col V led to an impaired tube formation ability of the HUVEC. Numbers indicate the quantification of the expression.

In further experiments the effects of exogenous Col V on tube formation was analyzed. For this purposes tube formation assays were performed in HUVEC grown on matrigel and exposed to CM of PSC, with or without Col V inhibition by siRNA (Reithmeier 2012). Medium of PCC could not promote the formation of tubes when either the normal standard medium was used nor the CM, whereas normal tube formation was observed in HUVEC grown in their standard medium. In HUVEC treated with CM of PSC, tube formation could be observed. This effect was reversed when HUVEC were treated with CM of siRNA treated PSC (Figure 37).

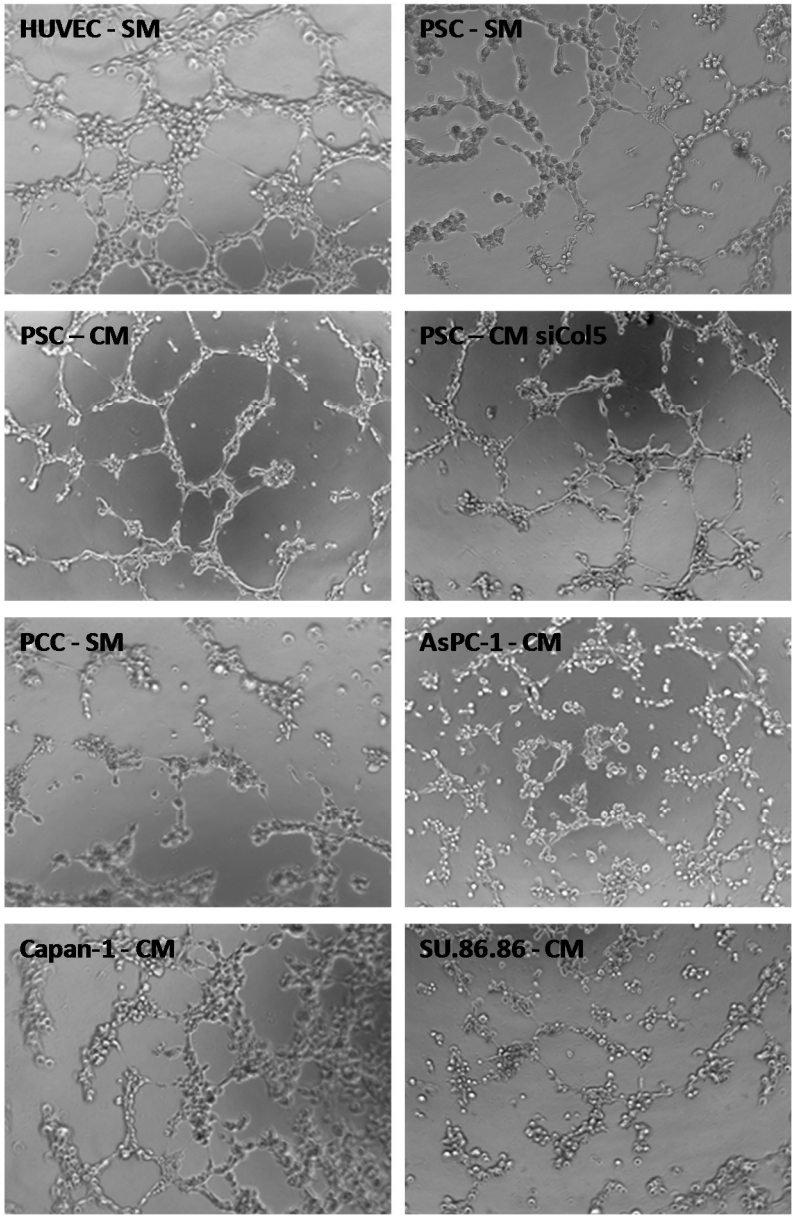

Figure 37: Tube formation of HUVEC treated with different CM. As control, HUVEC standard medium (HUVEC-SM) and standard medium of PSC (PSC-SM) was used. HUVEC treated with CM of PSC (PSC-CM) showed a good tube formation. Medium obtained from siCOL5 treated PSC (PSC-CMsiCOL5) led to an impaired tube formation. CM of the cancer cells was not promoting tube formation in all cases.

Despite the lack of evidence that Col V and angiogenesis correlate, the *in vitro* data showed that in general, PSC and, in particular, Col V influences the formation of new vessels.

3.8 Orthotopic mouse model of PDAC

The effect of PSC-derived Col V was tested additionally *in vivo* in an orthotopic mouse model. Therefore a stable knock-down of Col V in PSC was achieved by lentiviral transduction. Different constructs were used to obtain different knock-down efficiencies. Knock-down efficiency was tested using TaqMan assay and immunoblotting. To exclude unspecific side effects, transduction with a control vector was performed (shNT). A knock-down of 88 % (#10) or 75 % (#11) on mRNA level and of 29 % (#10) or of 61 % (#11) on protein level was achieved (Figure 38A and B) compared to the control vector. These two vectors were then used for stable transduction into PSC and subsequent implantation in nude mice. Protein extraction from primary tumors showed a knock-down of 40 % and 75 %, respectively (Figure 38C).

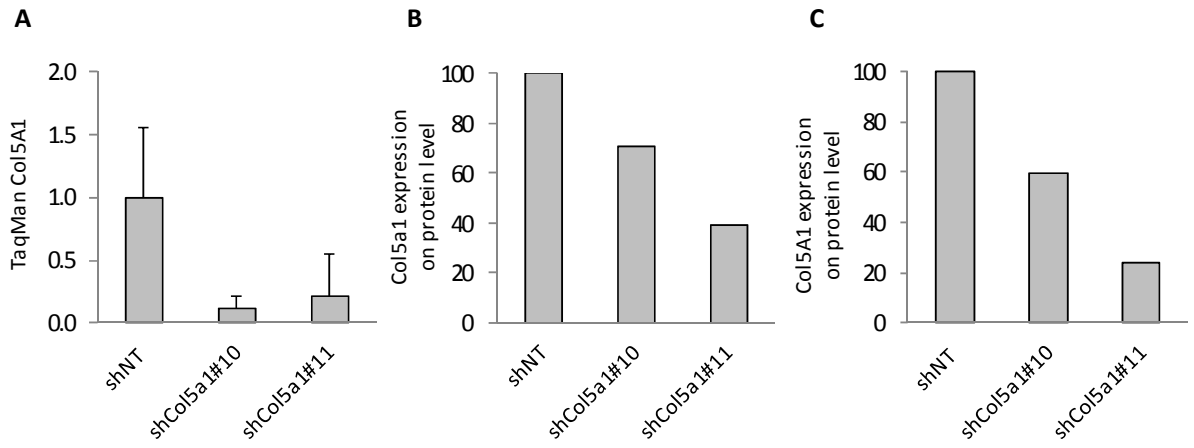

Figure 38: Efficiency of Col V knock-down in PSC with TaqMan assay (A) and immunoblotting (B) and in primary tumors with immunoblotting (C). (A) Knock-down of 88 % or 75 % was achieved on mRNA level. (B) Knock-down of 29 % was achieved with vector #10 and a knock-down of 61 % with the vector #11 on protein level. Protein extraction of the primary tumor showed that the knock-down is still present *in vivo* with a knock-down efficiency of 40 % and 75 %, respectively (C).

The tumors of mice that were implanted only with tumor cells showed a tendency towards smaller tumors (104.3 mm^3) compared to mice that were implanted with the mixture of cancer cells and PSC (191.8 mm^3) (Figure 39), which was not significant. Primary tumor growth was not significantly affected by the knock-down of Col V, but tumor volumes after transfection with vector #11 were slightly smaller compared to the control (158.0 mm^3).

Metastasis formation was investigated by counting the metastatic foci on the liver surface under the microscope. Although no difference in the tumor volume could be determined, a change in the metastasis formation was found. Mice implanted with PSC together with SU.86.86 had more metastases (38.6) compared to mice implanted with SU.86.86 cells alone (32.6). In comparison to that, mice implanted with the knock-down cells showed a reduced formation of metastatic sites with 8 metastases for vector #10 and 16.3 metastases for vector #11 (Figure 39).

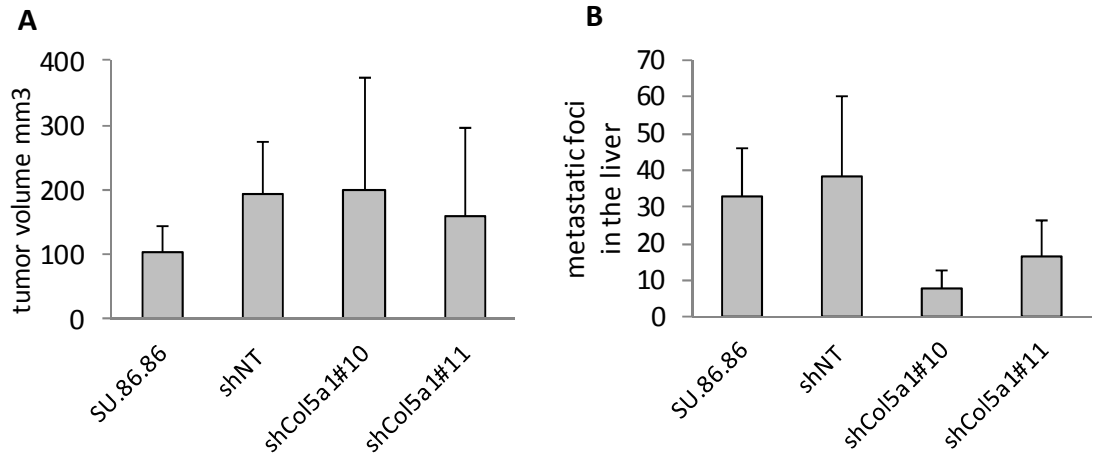

Figure 39: Tumor volumes (A) and metastatic foci on the liver surface (B) after 6 weeks. Mice implanted with PCC and PSC showed higher tumor volumes compared to mice implanted with PCC alone. Implantation of PCC together with PSC with a knock-down of Col V did only show a small decrease in the tumor volume, which was not significant (A). Metastatic formation on the liver surface was reduced in mice implanted with the PSC with a knock-down of Col V (B).

Upon morphologic analysis, tumor tissues were slightly infiltrated by inflammatory cells and central areas of necrosis occurred. Further, only a low stromal reaction was seen arising from the original pancreatic tissue and intra-tumoral (Figure 40).

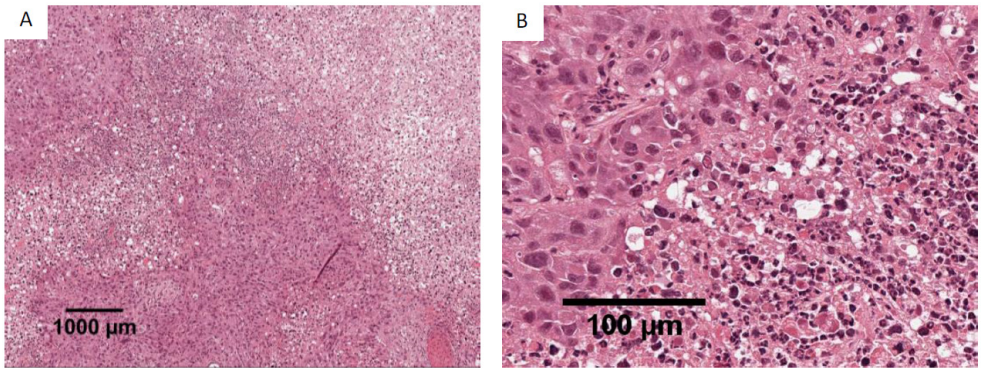

Figure 40: Representative HE stainings of primary tumors. Some necrotic areas (A) and inflammatory infiltrates (B) are seen in the HE staining.

To investigate if Col V knock-down was detectable at the level of protein expression and if transduced PSC retained their αSMA-positive phenotype *in vivo*, immunohistochemical staining of Col V and αSMA were performed. A reduced expression of Col V was clearly observed in the tumors after knock-down, whereas αSMA expression remained unchanged (Figure 41).

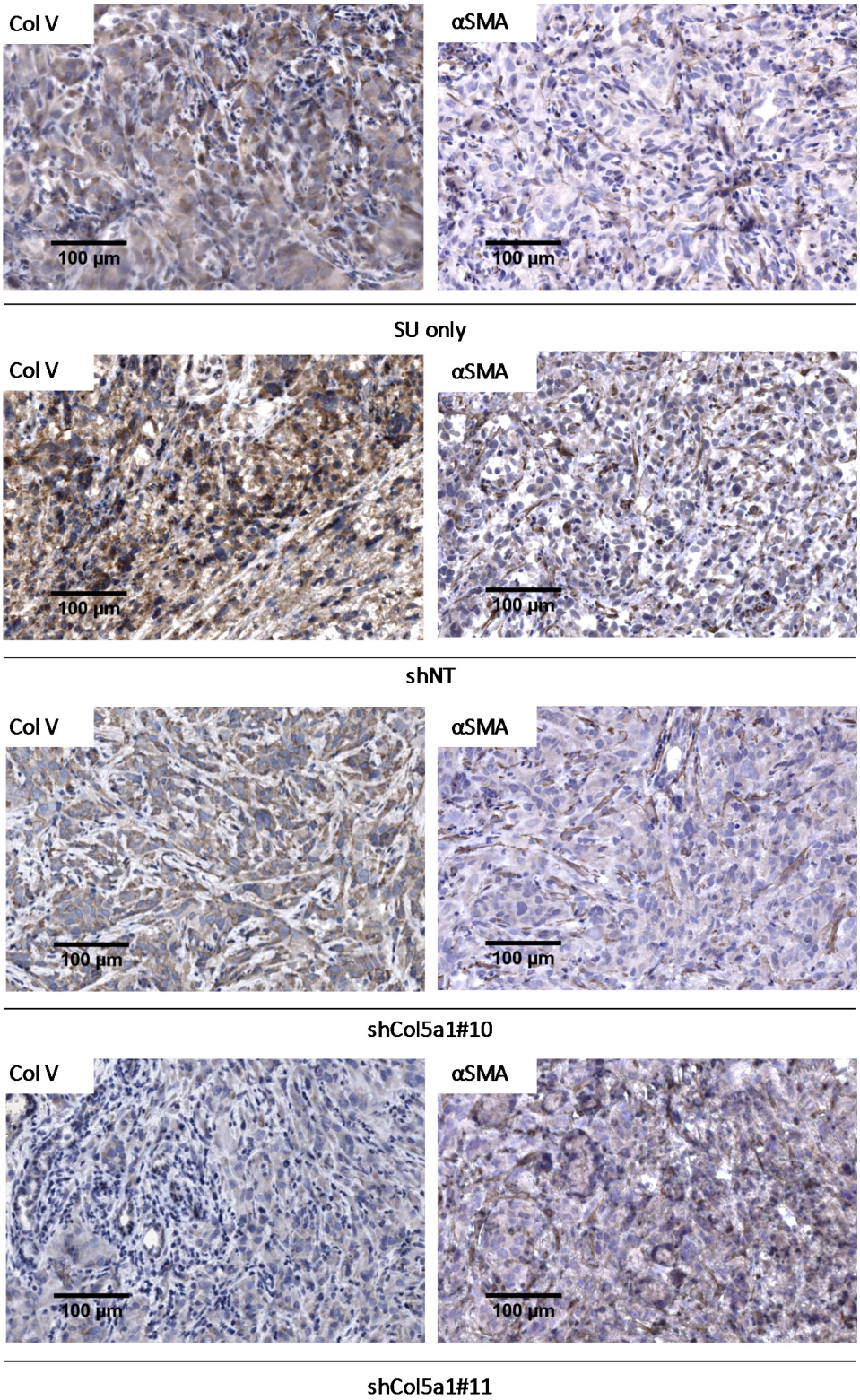

Figure 41: Representative Col V and αSMA stainings of primary tumor tissue. Col V staining is less intense in the tumors after knock-down whereas no change in the expression pattern of αSMA is observed.

To check if Col V influences neoangiogenesis *in vivo*, CD31 staining was performed and evaluated with software-based morphometric methods. Representative pictures of CD31 staining are shown in figure 42. Tumors of mice implanted with wild-type PSC showed a higher vessel density than tumors of mice implanted with altered PSC (Figure 43). Although the differences were not statistically significant, this result confirms the important role of Col V in angiogenesis.

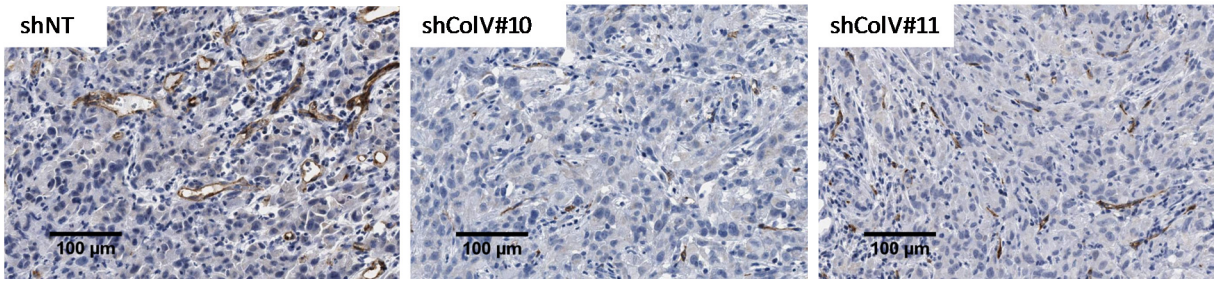

Figure 42: Angiogenesis in primary tumors. CD31 staining of tumors with the knock-down cells was less present compared to the controls.

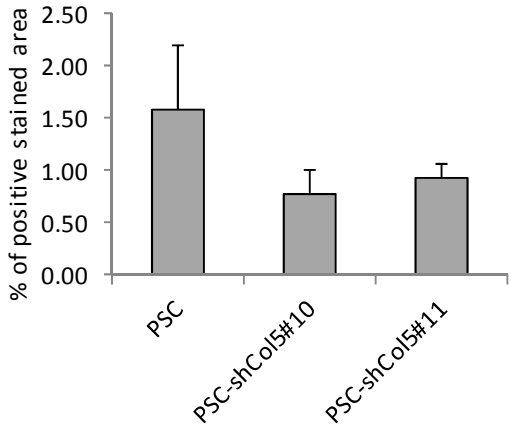

Figure 43: Positive stained area from tissue of primary tumor stained against CD31. Tumors of mice implanted with PSC with a stable Col V knock-down showed less formation of new vessels.

Taken together, PSC-derived Col V has an impact on metastatic formation *in vivo*. Tumors implanted with PSC with a Col V knock-down showed impaired formation of metastatic foci in the liver and reduced vessel densities in primary tumors.

4 Discussion

The development of an extensive stromal reaction, so-called desmoplasia, accompanies the progression of PDAC. The desmoplastic reaction consists of structural and cellular components (Binkley, Zhang et al. 2004). Among the structural proteins are various collagens, predominantly collagen type I (Apte, Park et al. 2004). Already in 1995 Imamura et al. showed a 3-fold increase of collagens in the stromal reaction accompanying PDAC compared to normal pancreatic tissue (Imamura, Iguchi et al. 1995). Among them, Col I and Col III have been extensively studied in PDAC. Col V, a more recently described ECM protein up-regulated in PDAC (Wehr, Furth et al. 2011), is known to contribute to the desmoplastic reaction of some tumor entities, such as breast cancer and colon cancer (Barsky, Rao et al. 1982, Fischer, Stenling et al. 2001). In the present study the functional role of Col V in pancreatic carcinogenesis was analyzed in detail.

4.1 Collagen type V expression during tumor progression

4.1.1 Collagen type V is increased in tumor progression

Upon immunohistochemical visualization of Col V during multistep carcinogenesis in the pancreas an up-regulation of stromal Col V expression with a shift in the expression pattern was found in the progression from precursor lesions to invasive cancer. In the PanIN lesions a basement-membrane like pattern was observed, in agreement with the known function of Col V as an important component of the *lamina densa* (together with Col IV) and of the *lamina fibroreticularis*, which is essential for the anchorage of the basement membrane to the stroma (Roll, Madri et al. 1980). Further, Adachi et al. described a linkage of the epithelium that grows on the basement membrane to the underlying connective tissue *via* Col V fibrils (Adachi and Hayashi 1994). In cancer tissues a more diffuse stromal expression was observed, in agreement with the disruption of the basement membrane and the loss of polarization, which characterizes invasive growth. In contrast, no Col V staining could be observed in normal tissue.

This increasing expression of Col V beginning in the early phases of the carcinogenesis throughout cancer progression points towards a potential functional implication of Col V in stromal remodeling. The specific expression of Col V in diseased but not in normal tissue, its presence in early precursor lesions and its specific stromal expression in PDAC, suggest Col V as highly selective target for the diagnosis of early PDAC (Wan, Pantel et al. 2013).

4.1.2 Collagen type V is produced mainly by PSC

Stromal components in PDAC in general are mainly produced by PSC (Haber, Keogh et al. 1999) and in breast cancer Col V was found to be expressed by stromal cells as well (Barsky, Rao et al. 1982).
Consistent with these known facts, expression analysis of Col V in human pancreatic tissues and cell lines revealed PSC as the main source of Col V in pancreatic cancer. Additionally, the expression of Col V by PSC is in accordance with the observed localization of Col V in the basement membrane-like pattern found in the precursor lesions and the diffuse staining pattern in the invasive cancer: PSC are located in the periacinar space, as well as perivascular and periductal (Omary, Lugea et al. 2007) in normal tissue, while during PanIN progression, PSC re-localize around the lesions, where they are activated and start producing ECM proteins (Erkan 2013). In invasive cancer, the basement membrane is disrupted and PSC are distributed throughout the whole stroma, where they continue to express Col V. In contrast, expression in pancreatic cancer cells is negligible. These data strongly indicate PSC as the main producers of Col V in PDAC.
In general, the stromal component is known to promote tumor growth, angiogenesis, inflammation, and metastasis and several studies suggested a tumor promoting role of PSC and collagen type I in PDAC (Armstrong, Packham et al. 2004, Madar, Goldstein et al. 2013). However, Özdemir et al. recently showed in a mouse model that tumor progression is increased after depletion of the stromal components, therefore suggesting a protective impact of the stromal reaction (Ozdemir, Pentcheva-Hoang et al. 2014). Whether Col V has a protective or a supportive role will be discussed in the following sections.

4.2 The functional role of collagen type V

To further unravel the function of Col V in tumor progression, its role in different cells types, (stromal cells, epithelial cells and endothelial cells) was investigated.

4.2.1 Role of collagen type V in fibroblasts

Col V is an extracellular matrix protein in tumor tissue, but it is also present in normal tissues with mesenchymal compartments, as for example in the dermis (Kobayasi and Karlsmark 2006) where it is expressed by dermal fibroblasts (Olsen, Peltonen et al. 1989, Achterberg, Buscemi et al. 2014).
For detailed analysis of the stromal component, PSC were compared to normal dermal fibroblasts which express Col V in a moderate amount (Olsen, Peltonen et al. 1989) and to fibroblasts obtained from patients affected by the Ehlers-Danlos syndrome, bearing a Col V mutation (Wenstrup, Florer et al. 2000). Ehlers-Danlos fibroblasts produce Col V on nearly normal expression levels but in a non-functional manner, leading to atypical Col I fibrillogenesis (Zoppi, Gardella et al. 2004). The normal expression level of Col V in Ehlers-Danlos fibroblasts could be confirmed in this study. In culture, they

show a spindle-like shape and can be identified through their expression of αSMA. We could show that Col V exerts autocrine effects on all types of stromal fibroblasts. Ehlers-Danlos-fibroblasts had impaired migration abilities which could be rescued by functional exogenous Col V, as previously shown (Viglio, Zoppi et al. 2008). The observed higher adhesion rates of PSC and the higher proliferation and migration rates of both HDF and PSC on standard substrate, as compared to Ehlers-Danlos-fibroblasts, underscore the functional importance of an intact Col V in stromal cells. Interestingly, endogenous and exogenous Col V affected PSC more strongly than other fibroblast types. PSC showed in fact the highest rates of adhesion, proliferation and migration in all settings, indicating a positive autocrine activation loop caused by Col V in the stroma of PDAC.

Taken together, the effects on stromal cells indicate that Col V is a decisive factor for relevant properties of stromal cells and it drives the activated phenotype of the cancer-associated fibroblasts.

4.2.2 Collagen type V signaling in epithelial cells is mediated through β1-integrin

In a further approach, the role of mesenchymal cell-derived Col V on epithelial cells and its role during pancreatic carcinogenesis were investigated. For this purpose, the signaling pathway downstream of Col V was analyzed. Most collagens, including Col V bind the major collagen receptors integrin $\alpha_2\beta_1$. Other integrins, such as $\alpha_1\beta_1$-integrin bind rather specifically to certain collagens, such as a collagen type IV and XIII (Ruggiero, Champliaud et al. 1994, Zoppi, Gardella et al. 2004). Upon binding of their respective binding partner, integrins cluster and associate with the cytoskeleton, as well as with signaling complexes thereby promoting assembly of actin filaments (Giancotti and Ruoslahti 1999). Examples for proteins activated by integrin ligand binding are the focal adhesion kinase (FAK), integrin-linked kinase (ILK) and Src kinase (Vuori 1998). Additionally, β_1-integrin is known to play an essential role in adhesion and invasion of PCC. Arao et al. showed that different cancer cells have different constitutive activities of β_1-integrin and this goes along with a higher invasive potential (Arao, Masumoto et al. 2000).

We could show a basolateral expression of β_1-integrin in the epithelial cells of early precursor lesions. In the context of the above mentioned finding that the expression of Col V is present in low-grade PanIN, an interaction of both molecules already in early phases of pancreatic progression seems likely. This interaction of Col V and β_1-integrin could mediate the anchorage of epithelial cells to the basement membrane and further enable epithelial downstream signaling of Col V at the PanIN stage. The loss of the distinct perilesional Col V expression pattern along with the progression from low-to high-grade PanIN, where a more apical staining is observed, suggests the loosening of the basement membrane during carcinogenesis, allowing the cancer cells to invade. Accordingly, Col V seems to be rather supportive than protective in PDAC.

Integrin β_1 signaling mediates functional processes like the adhesion of epithelial cells to the basement membrane, but also tumor progression, metastasis and angiogenesis (Li, Zhang et al. 2005). In breast cancer, β_1-integrin has been therefore recently suggested as a target for immunotherapy (Park, Zhang et al. 2008)**.** Although most studies focus on the β_1-integrin subunit (Schwartz 2001), van Slambrouck et al. showed that also α_2-integrin is responsible for the activation of the downstream signaling (Van Slambrouck, Jenkins et al. 2009). Blocking specifically this subunit resulted in poor adhesion, inhibited invasion and has recently been shown to be responsible for FAK-mediated ERK activation (Sawhney, Cookson et al. 2006).

In our experiments, the cell line SU.86.86 showed an increase in β_1-integrin expression when grown on Col V, indicating a potential autocrine influence of the signaling pathway. This upregulation of β_1-integrin could be explained by an increased trafficking of integrins, which is an important mechanism in adherent cells for adhesion, migration and invasion. This trafficking includes the delivery of newly synthesized integrins, integrin internalization and recycling (Caswell, Vadrevu et al. 2009, Margadant, Monsuur et al. 2011). Exogenous Col V alone does not seem to be able to trigger this trafficking, since the levels of β_1-integrin remained unchanged in the other cancer cell lines. Stromal cells themselves show β_1-integrin expression, indicating a potential involvement of this pathway also in this cell type. The changing expression pattern and upregulation of β_1-integrin during the carcinogenic process represents the basis for the subsequent activation of the downstream targets.

4.2.3 Downstream signaling is mediated through FAK and PAX

Downstream targets of β_1-integrin are FAK and PAX. FAK is a kinase that binds to the cytoplasmic domain of β_1-integrin (Schaller, Otey et al. 1995). Through the clustering of integrins, FAK gets autophosphorylated at the phosphorylation site pFAK-Y397 which correlates with an increased catalytic activity (Lipfert, Haimovich et al. 1992, Schaller, Hildebrand et al. 1994, Calalb, Polte et al. 1995) and in turn is important for adhesion (Calalb, Polte et al. 1995, Owen, Ruest et al. 1999). The second downstream signaling molecule investigated was PAX, which acts as a docking partner in adhesion (Schaller, Otey et al. 1995). Murasawa et al. showed Col V binding to β_1-integrin and downstream signaling through PAX and FAK and that β_1-integrin co-localized with pPAX-Y118 and pFAK-Y861 at the leading migration edge of the glomerular endothelial cells (Murasawa, Hayashi et al. 2008). Other types of collagens show an activation of similar pathways, e.g. collagen type IV activates the downstream phosphorylation of FAK and PAX in lung cancer cell lines (Mukhopadhyay, Gordon et al. 2005).

An activation of this pathway was seen in this study using a Col V-rich matrix. With immunoblot analysis the activation of the downstream targets FAK and PAX was verified in three of four cancer cell lines.

This sets the precondition for functional effects that stromal Col V might have on pancreatic cancer cell behavior mediating the carcinogenic processes.

4.2.4 Collagen type V-mediated β1-integrin signaling promotes functional *in vitro* effects of PCC

Carcinogenesis is a multistep process (Hanahan and Weinberg 2011), which can be investigated *in vitro* using different approaches. Adhesion to non-cellular surroundings is one of the first steps initializing carcinogenesis and furthermore cell-matrix interactions are prerequisites for cell dissemination (Crowe and Shuler 1999). Another important characteristic of a cancer cell is its uncontrolled ability to proliferate, while in normal tissue cell cycle progression is strictly controlled (Hanahan and Weinberg 2011). In addition, the desmoplastic reaction of PDAC contributes to therapy resistance (Neesse, Michl et al. 2011). In later stages, the formation of metastases takes place ultimately leading to systemic disease spread and often associated with death of the patients (Hanahan and Weinberg 2011).

The influence of Col V on these carcinogenesis-promoting cellular properties, namely adhesion, proliferation, cell migration and chemoresistance, were investigated using a Col V-enriched matrix. Adhesion, proliferation and migration were significantly promoted by this Col V-enriched matrix in addition to formation of lamellipodia, which are a prerequisite for migration (Cramer 1997). This substantiates the pro-migrative role of Col V. Using viability assays, it was shown that cancer cells grown on Col V had a significant higher survival rate after chemotherapeutic treatment, suggesting a protective role of Col V for the cancer cells and therefore worsening the treatment failure of pancreatic cancer. Chemotherapeutics do not only suppress cancer cell growth, they also can induce apoptosis, which in turn leads to cancer cell death. However, treatment failure is often due to the gain of resistance against apoptosis which is mediated through the upregulation of apoptosis inhibitors (Shi, Liu et al. 2002).

Analysis of the effects of Col V on apoptosis and cell cycle progression showed that apoptosis in this setting plays a minor role, whereas the cells grown on Col V proliferate in higher rates than cells on control plates, either treated with chemotherapeutics or not. Col V therefore promotes survival of PCC through increased proliferation rates rather than resistance against apoptosis.

Taken together, in this study three of four tested cell lines were functionally influenced by the Col V-enriched matrix in adhesion, proliferation, migration and chemoresistance. PANC-1 is the only cell line tested that was not influenced by Col V as *substratum*, neither in the activation of the pathway nor in subsequent *in vitro* assays. However, PANC-1 cells are derived from a poorly differentiated carcinoma and might therefore already be independent from certain ECM-derived signals. In comparison, AsPC-1, Su86.86 or Capan-1, which are moderately and well differentiated cell lines, respectively, responded to Col V stimulation. Additionally, SU.86.86 is the only described cell line that expresses Col V at low

levels, so that this cell line could exhibit some autocrine positive feedback loops, which further increase the influence of exogenous Col V. The different behaviors of the cancer cell lines reflect the fact that in every cancer entity an inter- and intra-tumoral heterogeneity is present, which might lead to different effects in different parts of the same tumor. This heterogeneity, which relates to a large variety of subpopulations of cells with different genotypes and phenotypes, is a major problem in the treatment of tumor patients and further impacts the treatment outcome (Fisher, Pusztai et al. 2013).

These functional findings for Col V described in this study are in accordance with observations reported in literature for other collagens, namely type I, III and IV, which were found to increase the malignant phenotype of pancreatic cancer cells. In detail, Collagen type I, another fibril-forming collagen, promotes the malignant phenotype of PCC (Armstrong, Packham et al. 2004). PCC on collagen type III show enhanced proliferation and cell migration compared to cells grown on plastic (Menke, Philippi et al. 2001). Collagen type IV promotes pancreatic cancer cell proliferation, migration and inhibits apoptosis (Ohlund, Franklin et al. 2013). The involvement of the β_1-integrin signaling pathway on these tumor promoting functions is also well described in literature. For example, the involvement of FAK on functional properties of PCC is already demonstrated, as a downregulation of FAK leads to an impaired adhesion and invasion (Sawai, Okada et al. 2005). FAK-deficient cells show impaired spreading on different ECM molecules and migrate poorly (Ilic, Furuta et al. 1995), but reconstitution with wildtype FAK could restore cell migration (Owen, Ruest et al. 1999, Sieg, Hauck et al. 1999, Sieg, Hauck et al. 2000) and overexpression of FAK in Chinese hamster ovary (CHO) cells enhances cell migration (Cary, Chang et al. 1996). Further, integrin β_1-mediated signaling directly increases adhesion, proliferation and migration (Grzesiak and Bouvet 2006).

As a proof of concept, blocking of the β_1-integrin pathway was achieved using two different inhibitors. In a first approach, disruption of integrin signaling was accomplished using the Src kinase inhibitor AZD0530 (AZD), which blocks phosphorylation of FAK and PAX (Green, Fennell et al. 2009) as it prevents the binding of the Src kinase to FAK (Bellis, Miller et al. 1995, Schaller and Parsons 1995). Accordingly, Col V mediated activation of β_1-integrin cannot be transmitted downstream. In a second approach, β_1-integrin was directly targeted by a blocking antibody (Mukhopadhyay, Gilchrist et al. 2004). Thereby, a more effective blocking of this particular pathway could be achieved, as the binding of Col V to its receptor is prevented. Efficient blocking of the signaling pathway by both approaches was confirmed by immunoblotting. This diminished Col V-induced adhesion, proliferation and migration in most tested cell culture systems. However, SU.86.86 cells showed less effective reduction of Col V-induced adhesion and proliferation upon inhibitor treatment, which might be attributed to additional endogenous Col V expression in these cells.

PSC-induced chemoresistance could be shown to be reversible upon inhibition of β_1-integrin *via* a blocking antibody and siRNA (Mantoni, Lunardi et al. 2011). In the present study, it could be shown

that Col V is a single PSC-derived factor promoting chemoresistance, as blocking of β_1-integrin signaling completely reversed Col V-mediated chemoresistance. Again, SU.86.86 cells reacted less strongly to inhibitor treatment substantiating the theory that endogenous Col V expression influences this cell line.

Even upon inhibitor treatment, the effects of Col V on adhesion, proliferation and migration were not entirely blocked, but only significantly reduced. Accordingly, immunoblotting showed that the inhibitors did not completely block the signaling pathway. Thus, residual levels of downstream signaling remained enabled, possibly through the activation of other pathways, like the NG2 (neural/glial antigen 2) pathway (Murasawa, Hayashi et al. 2008).

These results prove the involvement of the β_1-integrin signaling pathway, concerning cancer promoting characteristics induced by stromal factors in general and by Col V in particular and indicate that the disruption of the stromal reaction might be a potential therapy approach. These results further underline the significance of Col V during the carcinogenesis in which Col V is already present from the very beginning and thereby promoting the activation of the β_1-integrin downstream targets.

4.2.5 Collagen type V affects angiogenic processes

In addition to the autocrine functions on PSC and functions in mesenchymal-epithelial interactions during PDAC development described in this study, the role of Col V in endothelial cells was investigated.

Col V is known to be important for the wound healing and this process is impaired in patients with Ehlers-Danlos syndrome (Beighton, De Paepe et al. 1998). Angiogenic processes are very important features in cancer, as tumors need to be provided with nutrients and oxygen (Hanahan and Weinberg 2011). Tumors therefore induce neo-angiogenesis, which remains activated, hence always further promoting tumor growth (Hanahan and Folkman 1996). A direct impact of the single factor Col V on angiogenesis in PDAC has not been described so far.

Investigating angiogenesis in tumor tissue, a correlation between Col V and CD34 expression could not be detected by semi-quantitative analysis. The drawback of the CD34 marker is that it stains not only endothelial progenitor cells but also mature endothelial cells, so that we also relied on morphological criteria for the exclusion of mature vessels from the analysis. The lacking correlation of CD34 with Col V could be explained through the grade of hypoxia in the tumor tissue, where more angiogenesis takes place at the invasive front compared to the tumor center (Erkan et al. 2009). In this study, only the whole tumor tissue was evaluated without distinction between tumor center and periphery.

Nevertheless, an important role of Col V on angiogenesis was shown with tube formation assays, as tube formation of HUVEC was impaired by Col V knock-down, substantiating the important role of Col V in wound healing in general. The effect of Col V as growing substrate on the formation of tubes

was investigated and compared to the tube formation on collagen type I, which has already been described (Montanez, Casaroli-Marano et al. 2002). In comparison to the impaired tube formation after knock-down, tube formation was increased when HUVEC were grown on a Col V matrix but with less vessel formation compared to a Col I matrix, indicating that these proteins strengthen angiogenic processes.

The paracrine effect of Col V on angiogenesis could be demonstrated using supernatants of PSC for the tube formation assays, as CM from PSC lacking Col V reduced tube formation, indicating that also exogenous Col V secreted by PSC may influence vessel formation. No influence of PCC on tube formation was detected, indicating that PSC are a mainly responsible for the formation of new vessel in pancreatic cancer.

In vivo, the effects of Col V on angiogenesis obtained *in vitro* could be confirmed, as a reduced content of newly formed vessels was found in mice after implantation with PSC having a stable knock-down of Col V, shown by the smaller amount of CD31 positive areas. Angiogenesis is an important step in the metastatic formation and promotes tumor growth by supplying cancer cells with nutrients and providing the route for tumor cells to exit the primary tumor and to enter circulation (Folkman 2002). Previous studies already showed that the vascular density is a prognostic indicator of metastatic potential with a higher incidence of metastasis in highly vascular tumors (Zetter 1998). Col V, though it was not correlated to the microvessel density, was shown to be important for the angiogenic processes *in vitro* and neo-angiogenesis *in vivo*, sustaining the important role of Col V in tumor angiogenesis and further in the metastatic potential.

4.3 Crosstalk between PSC and PCC is influenced through collagen type V

In this study it was found that Col V is expressed by PSC and can directly influence this cell type. In addition, Col V influences the cancer cells *via* promoting an aggressive phenotype and impacts angiogenic processes. Crosstalk between PSC and PCC in PDAC is a well-known fact (Vonlaufen, Joshi et al. 2008). The role of Col V in this process is of great interest in order to elucidate the influence of Col V on the tumor progression in the outlook of targeting this protein as therapeutic option.

Targeting Col V with a transient knock-down altered the morphology of PSC without affecting their activation status, as shown by an unchanged expression of αSMA. Cytoskeleton arrangement is usually mediated through the binding of ECM proteins to different surface receptors (Geiger, Bershadsky et al. 2001). Col V therefore seems to be one of the ECM proteins mediating the cytoskeleton arrangement without affecting the activation status *per se*.

Functionally, cancer cells showed a decreased proliferation and invasion when treated with the supernatant of PSC with a Col V knock-down. That this effect was achieved only in moderately differentiated cell lines could be explained by the fact that these cell lines can be still more influenced

by exogenous signals than poorly differentiated cell lines. However, Col V as growth substrate has stronger influences on the cancer cells than soluble Col V in the supernatant. This could be explained with the physiological role of Col V as structural component of the ECM. Additionally, the cells have probably less contact to soluble Col V, which further diminishes the effects. Similar results were shown by investigating the effects tenascin-C, another important ECM protein, in PDAC (Paron, Berchtold et al. 2011). On the other hand, PSC-derived Col V secretion was increased when PSC were treated with supernatants of PCC, while the amount of Col V in the cell lysates was equal, indicating an influence in the processing and release rather than in the expression *per se* and suggesting paracrine interactions between PSC and pancreatic cancer cells. Similar effects are described for Col I, whose expression in PSC can be enhanced using supernatants of different cancer cell lines (Bachem, Schunemann et al. 2005).

As Col V showed tumor promoting effects in *in vitro* conditions, it was analyzed if a knock-down of Col V can reverse this effect *in vivo*. The orthotopic model of pancreatic cancer, with the implantation of PSC together with PCC was already performed by several groups with different cancer cell lines (Hwang, Moore et al. 2008, Vonlaufen, Joshi et al. 2008, Xu, Vonlaufen et al. 2010). These groups showed that PSC promote tumor growth and formation of metastases. As a transient knock-down does not last the time needed for *in vivo* experiments, a stable knock-down of Col V was achieved using lentiviral transduction. Non-immortalized PSC were already effectively transduced with lentivirus previously (Tang, Yuan et al. 2012). The fact that the knock-down on RNA level was more prominent than on protein level could be probably due to the ability of the cells to regulate Col V post-transcriptional and therefore accumulate the protein. Higher tumor volumes were found for the mice implanted with cancer cells and PSC together, than for mice implanted with cancer cells alone. However, in this case the tumor volumes showed a high degree of heterogeneity. During the operation, when the pressure inside the pancreas is high, some cells inevitably flow out from the pancreas. These cells could adhere to the peritoneum and the liver. Although the operation was executed as standardized as possible, some tumor cells were probably able to adhere more efficiently due to interindividual differences between mice and therefore were able to promote more neo-angiogenesis and in turn a faster tumor growth.

With this experiment we could confirm the effects of Col V on the invasive ability of cancer cells, which was already shown *in vitro*. The lower microvessel density found for mice implanted with the Col V-deprived PSC might be one reason for the lower metastatic potential, since an adequate blood supply is important for the migration of the cells (Folkman 2002).

It could be shown in this thesis that Col V clearly plays an important role in pancreatic cancer progression. On the one hand, PSC-derived Col V promotes the malignant phenotype of PCC and PCC increase the secretion of Col V in PSC, indicating a positive feedback loop in this system. Taken

together, this supports the hypothesis that Col V, as a molecule of the ECM, plays an important role in the complex interaction processes between PCC and PSC concerning the malignancy of PCC and the secretion of ECM proteins.

4.4 Collagen type V in therapy

Pancreatic cancer is one of the gastrointestinal tumors with the highest resistance to chemotherapy (Melisi and Budillon 2012). In the last years different approaches like surgery, radiation and chemotherapy had little effects in improving patients' survival. As the tumor can consist of 80 % of stromal desmoplasia (Erkan, Hausmann et al. 2012), the depletion of the desmoplastic stroma therefore seems to be a promising tool in treatment of PDAC, as it acts as a barrier for chemotherapeutics (Neesse, Michl et al. 2011). Col V, found already early in tumor progression, could not only be used for the early detection, it could also serve as potential target to deplete structural stromal components because of its strong expression in the tumor tissue.

Recent findings indicate that the depletion of the stroma has to be done with caution, as this can have opposite effects (Ozdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014). For example, Özdemir et al. found that the depletion of the stroma increased tumor progression and metastasis formation. However, in this study complete depletion of PSC was achieved, implying huge stress to cancer cells, which in turn might promote the aggressive phenotype of the cancer cells. In contrast, depletion of a factor like Col V could lead to a more favorable outcome, as removal of a critical structural component might promote loosening of the dense stromal reaction. This would in turn lead to a less hypoxic microenvironment and thus to a better drug delivery. This again indicates that combination therapies of a potential anti-Col V drug and chemotherapy could be an advantageous option for the treatment of PDAC.

4.5 Conclusion

This study provides essential new knowledge concerning the functional relevance of Col V on PSC, tumor cells and intra-tumoral angiogenesis in a tumor-supportive manner. The early induction of Col V and its sustained expression during tumor progression has an effect on all tissue components. Col V mutation, as it occurs in the Ehlers-Danlos syndrome, showed that an intact endogenous Col V is important for fibroblasts' properties, like adhesion, proliferation and migration. Further, Col V activated the β_1-integrin signaling pathway with its downstream targets FAK and PAX and affected the adhesion, proliferation and migration of pancreatic cancer cells *in vitro*, while these effects were partially blocked through specific inhibitors. This pathway therefore represents a possible target for therapies with the background of destroying the stromal reaction. One very interesting fact found in this work is the influence of Col V on angiogenic processes. It was first found that Col V influences tube

formation *in vitro*. This fact was verified *in vivo* in an orthotopic mouse model, where tumors consisting of tumor cells and PSC with a Col V knock-down showed less formation of vessels in the primary tumor. This indicates an important aspect in the development of new therapy options. Further, *in vivo* a decreased invasive potential was found when Col V was downregulated. The described interaction between PSC and PCC were verified under the context of Col V, as an induction of the Col V secretion in PSC was found when treated with PCC and *vice versa*, PCC were influenced in their proliferation and invasion when PSC-derived Col V was lacking. The current study is an essential contribution to the understanding of early epithelial-mesenchymal interactions in PDAC development. Although stromal therapy has not been successful in the past, inhibition of stromal parts could bring the solution.

Abbreviations

µl	Microlitre
5-FU	5-Fluorouracil
Ab	Antibody
ADM	Acinar-ductal metaplasia
AFL	Atypical flat lesions
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
αSMA	α-smooth muscle actin
AT	Ataxia teleangiectatic
ATM	Ataxia telangiectasia mutated
AZD	Saracatinib
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
bp	Base pair
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BSA	Bovine serum albumin
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
CM	Conditioned medium
Col I	Collagen type I
Col III	Collagen type III
Col V	Collagen type V
ColF1	Fibrillar collagen C-terminal domain
CP	Chronic pancreatitis
Gem	Gemcitabin
Da	Dalton (1 Dalton = 1 g/mol)
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Complete Dulbecco's Modified Eagle's Medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphates
EBM	Endothelial basal medium
ECL	Enhanced chemoluminescence
ECM	Extracellular matrix
EDF	Ehlers-Danlos fibroblasts
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGM	Endothelial growth medium
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERBB2	human epidermal growth factor receptor 2
ERK1/2	Mitogen-activated protein kinase kinase
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FAMM	Familial atypical multiple mole melanoma
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FOBC	Familial breast and ovarian cancer
FPC	Familial pancreatic cancer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
Gem	Gemcitabine
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HDF	Human dermal fibroblasts
HNPCC	Hereditary nonpolyposis colorectal carcinoma
HP	Hereditary pancreatitis
HRP	Horseradish peroxidase
hrs	Hour(s)
HUVEC	Human umbilical vein endothelial cells
ICR	Interrupted collagenous region

IPMN	Intraductal papillary mucinous neoplasms
kb	Kilo-base pair
K-Ras	Kirsten rat sarcoma viral oncogene homolog
mA	milli Ampere
MAPK	Mitogen-activated protein kinase
MCN	Mucinous cystic neoplasm
min	Minutes
ml	Milliliter
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
mM	Millimolar
MMP	Matrix metalloprotease
mRNA	Messenger RNA
MSH2	MutS protein homolog 2
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NG2	Neuroglycan 2
NHR	Nonhelical region
p16	Cyclin-dependent kinase inhibitor 2A
PanIN	Pancreatic intraepithelial neoplasia
Pax	Paxillin
PAX	Paxillin
PBS	Phosphate buffered saline
PCC	Pancreatic cancer cells
PCPE-1	Procollagen C-endopeptidase enhancer 1
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
pFAK-576/577	Focal adhesion kinase phosphorylated at tyrosine 576/577
pFAK-Y397	Focal adhesion kinase phosphorylated at tyrosine 397
pFAK-Y861	Focal adhesion kinase phosphorylated at tyrosine 861
PI3K	Phosphatidylinositide 3-kinase
PJS	Peutz-Jeghers syndrome
pPAX-Y118	Paxillin phosphorylated at tyrosine 118
PRSS1	Trypsin-1
PSC	Pancreatic stellate cells
Rho-ROCK	Rho-associated protein kinase

RNA	Ribonucleic acid
rpm	Rounds per minute
rRNA	Ribosomal RNA
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfat-polyacrylamide gel electrophoresis
sec	Seconds
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SMAD4	Mothers against decapentaplegic homolog 4
SN	Supernatant
SPARC	Secreted protein acidic and rich in cysteine
Src	Rous sarcoma
STK11	Serine/threonine kinase 11
TBS	Tris Buffered Saline
TC	Tubular complex
TEMED	Tetramethylethylenediamine
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
TIMP-1	Tissue inhibitor of metalloproteinases-1
TNFα	Tumor necrosis factor α
TP53	Gene coding for tumor protein p53
TPA	12-O-tetradecanoylphorbol-13-acetate
TRIS	Tris(hydroxymethyl)aminomethane
TSPN	Thrombospondin
TTBS	Tris-Buffered Saline + Tween 20
UV	Ultraviolet
V	Volt
VEGF	Vascular endothelial growth factor
X-gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid

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Publications

This thesis was prepared at the Technische Universität München, Institute of Pathology under the supervision of Prof. Irene Esposito and Prof. Michael Groll from March 2010 to September 2014.

Parts of this thesis have already been published as listed below:

Berchtold S, Grünwald B, Krüger A, Reithmeier A, Hähl T, Cheng T, Feuchtinger A, Born D, Erkan M, Kleeff J, Esposito I. Collagen type V promotes the malignant phenotype of pancreatic ductal adenocarcinoma. Cancer Lett. 2015 Jan 28; 356 (2 Pt B):721-32.

Acknowledgements

I would especially like to thank Prof. Esposito for giving me the opportunity to perform my Ph.D. Thesis in her group and offering me such an interesting topic. I am very grateful for her interest and enthusiasm in my project as well as her help and dedicated support through all the years. Her encouragement and her support created a productive and stimulating atmosphere, also extending beyond work.

Along these lines I also would like to thank Prof. Michael Groll for being my doctoral thesis supervisor.

A big thanks goes to my colleagues at the Institute of Pathology for the great time we had together, especially Susanne Haneder, Sandra Hiltensberger, Dr. Björn Konukiewitz and Katrin Lindner who were always there for me and became good friends. Dr. Katja Steiger, Dr. Melissa Schlitter and all my other colleagues for the profound discussions, good advice and a pleasant work experience.

I am very thankful for the excellent assistance of my master students and interns Anja Reithmeier, Kerstin Pflieger, Teresa Hähl and Marina Krummhaar as well as for the good times with my medical students Lena, Angi, Ralph and Anne-Christine. All of them have been a great asset to the whole group.

Further, I want to thank Dr. Annette Feuchtinger for her help with the Defininens Software, Dr. Wolfgang Beisker for his help with the FACS analysis and Dr. Tao Cheng for the orthotopic implantation. Special thanks goes to Barbara Grünwald for her enormous assistance during the animal experiment, all the fruitful discussions we had about my work, but even more for her friendship and her help in difficult times.

And last but definitely not least, I would like to thank my parents, my sister Alex and my friends who always helped me in good and bad times and to Basti for all the years of support. Finally, my special thanks goes to Basti for pushing me, his patience and his loving support!

Declaration

I, Sonja Berchtold, hereby declare that I independently prepared the present thesis, using only the references and resources stated. This work has not been submitted to any examination board yet. Parts of this work have been or will be published in scientific journals.

Erklärung

Hiermit erkläre ich, Sonja Berchtold, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die Arbeit wurde noch keiner Prüfungskommission vorgelegt. Teile dieser Arbeit wurden bzw. werden in wissenschaftlichen Journalen veröffentlicht.

Sonja Berchtold
München, September 2014