A fluorescence microscopy image showing a complex network of green and red fluorescent signals against a dark background. The green signals form a dense, interconnected web, while the red signals appear as distinct, bright clusters or spots, likely representing specific cellular components or markers within the tumor stroma.

Targeting and Modulation of the Tumor Stroma in Pancreatic Cancer

Jonas Schnittert

*Targeting and Modulation of the Tumor Stroma in
Pancreatic Cancer*

JONAS SCHNITTERT

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TARGETING AND MODULATION OF THE TUMOR STROMA IN PANCREATIC CANCER

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
prof.dr. T.T.M. Palstra,
on account of the decision of the graduation committee,
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Dedicated to my family, friends and colleagues

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Chapter 1- General Introduction

1.1 Pancreatic ductal adenocarcinoma

The first description of pancreatic cancer was provided by Giovanni Battista Morgagni in 1761 ¹. The most common form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) ². Until today, PDAC has one of the poorest prognosis of all cancers with the lowest 5-year-relative survival rate (8 %), and presents an unsolved problem in cancer medicine ³. Each year, around 100,000 people in Europe and 50,000 people in the USA get diagnosed with PDAC ⁴. According to the American Cancer Society PDAC belongs, only in females, to the top 10 of estimated new cases for 2018, with 26,240 estimated cases in the USA. Strikingly, PDAC is ranked fourth for the number of estimated death in 2018 in males (behind lung & bronchus, prostate and colon & rectum) and females (behind lung & bronchus, breast and colon & rectum), with 23,020 and 21,310 new cases, respectively. However, PDAC is expected to become the second cause of cancer-related death by 2020 ⁵. Of the five cancers with the highest number of estimated death in males and females, identified by the American Cancer Society, PDAC has the worst prognosis in The Netherlands regarding the survival rate (Figure 1).

Figure 1. Survival rate in percent for prostate, breast, colon & rectum, lung and pancreatic cancer in The Netherlands. Pancreatic cancer shows the poorest survival rate in the Netherlands for the period from 2011 – 2015 ⁶.

1.2 Diagnosis and risk factors

The risk to develop PDAC over a lifetime is 1.49 %⁷. Most PDAC incidences occur between 60 and 80 years of age⁵. The symptoms of PDAC become stronger with the development of the disease and include mid-epigastric pain, weight loss, malaise, nausea and fatigue⁵.

The diagnosis of PDAC is mostly dependent on the symptoms of the disease, which only appear late during disease progression. Therefore, around 50 % of patients with PDAC present with locally advanced or metastatic disease at the time of diagnosis, with the main metastatic sites including the liver, lungs and peritoneum⁷. Therefore, most PDACs are diagnosed when there is no longer any chance for cure. Early diagnosis might be possible in patients with an increased risk to develop PDAC or a family history of PDAC⁵. It is estimated that around 90 % of all PDAC cases are sporadic while the other 10 % are hereditary⁵. The main environmental risk factors for sporadic PDAC are tobacco smoking, obesity, long standing diabetes mellitus and alcohol induced pancreatitis⁸⁻¹¹. For hereditary PDAC patients might present genetic alterations. Mutations known to be associated with PDAC include STK11 mutation recognized in Peutz-Jeghers syndrome, the cationic trypsinogen gene PRSS1 and SPINK1 mutations associated with hereditary pancreatitis, the p16 mutation linked to familial atypical multiple mole melanoma (FAMMM), the adenomatous polyposis coli (APC) mutation responsible for familial adenomatous polypois (FAP), BRCA1 and BRCA2 mutations increasing the risk for hereditary breast ovary cancer (HBOC), MLH1 and MSH2 gene mutations associated with hereditary nonpolyposis colon cancer (HNPCC), and the ataxia telangiectasia (ATM) mutation⁵.

Generally, the examination of pancreatic cancer is performed by computer tomography (CT) and in uncertain cases magnet resonance imaging (MRI) and endoscopic ultrasound (EUS) may be used to image ductal structures⁵. In accordance with the International Cancer of Pancreas (CAPS) Consortium, patients presenting genetic risk factors or a family history of pancreatic cancer should be initially screened with EUS or MRI/magnetic resonance cholangiopancreatography¹². However, more cost effective and practical screening methods like blood biomarkers are required for PDAC. The Sialyl Lewis carbohydrate antigen (CA 19-9) is the only clinical serum biomarker for PDAC with a sensitivity and specificity of around 80 %. However, the sensitivity is lowered in early PDAC and therefore CA 19-9 is not applicable for screening and currently only used for therapeutic monitoring^{13,14}. The search for novel

biomarkers for PDAC is an intense field of research. The detection of circulating tumor cells ¹⁵⁻¹⁷ or circulating tumor DNA ^{18,19} from liquid biopsies have the potential to be effective tools in the prognosis of pancreatic cancer.

1.3 Standard treatment of PDAC

Whenever possible PDAC patients are treated with a surgical resection of the tumor. The preoperative evaluation of PDAC patients is based on the assessment of the relationship between the tumor and the major adjacent vessels, portal vein (PV), superior mesenteric vein (SMV), superior mesenteric artery (SMA), celiac axis (CA), hepatic artery (HA), and the presence of metastasis ⁵. The respectability of PCAD is dependent on the absence of distant metastasis, the lack of SMV or PV distortion and clear fat planes around the CA, HA and SMA ²⁰. Additionally, the consideration for pancreatic resection involves a risk-benefit assessment ².

The majority of PDAC cases are metastatic at the time of diagnosis and a surgical resection of these tumors is not feasible. Therefore, patients with metastatic PDAC are mostly treated with chemotherapy with the aim to reduce symptoms and prolong life. Fluorouracil (5-FU) has been the main standard of care but was replaced by the superior gemcitabine ²¹. Next, a combination therapy of gemcitabine together with the EGFR inhibitor, erlotinib, resulted in a significant increase in survival time from 5.9 to 6.2 month but added toxicity ²². In a randomized clinical trial, FOLFIRINOX was compared to gemcitabine and increased survival from 6.8 to 11.1 month ²³. Additionally, survival benefit compared to gemcitabine was achieved by the combination of gemcitabine with nab-paclitaxel (Abraxane), improving the survival time from 6.7 to 8.5 month ²⁴. Both FOLFIRINOX and Abraxane showed toxic side effects compared to gemcitabine ^{23,24} but are currently golden standard treatments for PDAC in patients able to tolerate the side effects.

1.4 Pathophysiology

Anatomically, the pancreas is divided into the exocrine pancreas composed of acinar cells, centroacinar cells and ductal cells, responsible for food absorption and the endocrine pancreas, consisting of the Islets of Langerhans that secrete the hormones insulin and glucagon for glucose and lipid homeostasis. With 85-90% PDAC is the most common form of pancreatic cancer ^{25,26}. Within invasive PDAC, common genetic mutations include mutational activation of the oncogene KRAS and inactivation the three tumor suppressor genes: *TP53*,

p16/CDKN2A, *Smad4* and *BRCA2* ^{27,28}. Additional genetic abnormalities in invasive PDAC include widespread chromosomal losses, gene amplifications and telomere shortening which is believed to contribute to chromosomal instability ²⁶.

PDAC develops from histologically different precursor lesions known as pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN), with decreasing frequency of development, respectively ²⁹. The majority of invasive PDAC develops from PanIN lesions (Figure 2) ²⁶. These lesions are characterized by their degree of dysplasia, into PanIN-1, PanIN-2 and PanIN-3 (Figure 2) ³⁰. Early detection of PanIN lesions present the opportunity to cure pancreatic cancer before the development of an invasive carcinoma ²⁷. Genetic analysis of PanIN lesions has shown, that PanIN-1 lesions already show mutations of *KRAS*, *p16/CDKN2A* and *BRAF* ^{31,32}. These findings indicate the earliest genetic events driving pancreatic neoplasia ²⁷. Next to PanIN, IPMN lesions are precursors of invasive PDAC ²¹. Early detection of IPMN lesions could also present the opportunity to cure pancreatic cancer before the development of an invasive carcinoma. Within 95 % of these lesions, genetic mutations of *KRAS* and/or *GNAS* were found ³³.

Figure 2. Graphical model for the progression of PDAC from a normal cell to metastatic PDAC, indicating genetic mutation associated with PanIN precursor lesions. The figure was adapted from Iacobuzio-Donahue et al. ²⁷.

1.5 The pancreatic tumor stroma

PDAC presents with a highly abundant desmoplastic reaction which is also known as the pancreatic tumor microenvironment (TME) or pancreatic tumor stroma. The pancreatic tumor stroma can occupy up to 90% of the entire tumor volume³⁴. The tumor stroma is composed of non-cancerous cells, including cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), pericytes, endothelial cells, and infiltrating immune cells (e.g. T-cells, natural killer (NK) cells and macrophages), ECM proteins (e.g. collagen and fibronectin) and soluble factors, including cytokines and growth factors³⁵. CAFs have been recognized as the most prominent cell type in the tumor stroma of pancreatic cancer³⁶⁻³⁸. Recently, the cellular components, and especially CAFs of the stroma were identified as drivers of tumorigenesis and their role in promoting tumor growth, angiogenesis, invasion and metastasis^{35,37}.

Figure 3. A graphical depiction of the dense tumor stroma in pancreatic cancer. The depiction includes the most prominent cell types, CAFs, endothelial cells, pericytes, macrophages, NK-cells, T-cells, and the non-cellular component, ECM proteins (e.g. collagen, fibronectin) and soluble factors (growth factors, cytokines).

Pancreatic stellate cells which have first been described in 1982 by Watari *et al.*³⁹, have more recently been recognized as precursors of CAFs and their key role in producing the desmoplastic reaction in PDAC⁴⁰⁻⁴³. Recently, Öhlund *et al.* have made a first step in defining two different CAF populations by introducing

myofibroblastic and inflammatory CAFs as CAFs with a distinct function and location in the stroma of PDAC ⁴⁴.

1.6 Pancreatic stellate cells

Pancreatic stellate cells (PSCs) are star shaped stromal cells, located at the basolateral aspect of acinar cells or surround perivascular and periductal regions in the healthy pancreas, and were first isolated and cultured in 1998 ^{45,46}. The functions of quiescent hPSCs include the storage of lipid droplets rich in vitamin A, basic exocrine and endocrine secretion, phagocytosis and immunity ⁴⁷. During the development of PDAC, quiescent PSCs become activated via various mechanisms, including risk factors (ethanol and its metabolites, chronic inflammation and smoking), environmental stress (e.g. hypoperfusion, hypoxia, oxidative stress), cellular factors (e.g. IL-1, IL-6, HIF1 α , TGF- β , CCN2), and molecular regulating pathways (e.g. Wnt/ β -catenin signaling, PI3K pathway), resulting in a transformation of quiescent PSCs into cells with a activated myofibroblast-like phenotype ⁴⁸. Once activated, PSCs lose their cytoplasmic vitamin A storing lipid droplets and start to express CAF markers such as α -smooth muscle actin which serves as a marker for the identification of activated hPSCs and is also known as a negative prognostic factor in PDAC ^{48,49}. Furthermore, activated hPSCs are the most important precursors of CAFs ⁴⁸. When hPSCs are in the activated state, they acquire a proliferative phenotype and induce desmoplasia by synthesizing abundant extracellular matrix (ECM) components such as collagens, fibronectin, laminin and hyaluronic acid and unbalanced expression of MMPs and TIMPs (Figure 4) ^{43,48}. Additionally, activated PSCs have an increased secretion of cytokines such as interleukin-1, 6, 8 and 10 (IL-1, 6, 8 and 10) and growth factors, including insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) (Figure 4) ^{43,50,51}. These growth factors have the ability to promote angiogenesis and proliferation and migration of epithelial cancer cells, invasion and metastasis (Figure 4) ^{43,48,52}. Moreover, cancer cells secrete cytokines as IL-1, 6 and TNF- α , and growth factors including TGF- β 1, PDGF ⁵³. These interactions result in an active crosstalk between cancer cells and activated hPSCs (Figure 4).

Figure 4. Schematic depiction showing hPSC activation and the interaction between hPSCs and pancreatic cancer cells, contributing to cancer progression.

1.7 Role of hPSCs in tumor progression

Within the tumor stroma growth factors, chemokines, cytokines, miRNAs and exosomes secreted by PSCs are known for their ability to act in an autocrine fashion, resulting in PSC activation or exert paracrine signals on epithelial tumor cells to increase the proliferation, migration, and invasion of tumor cells⁵⁴⁻⁵⁸. Additionally, paracrine factors secreted by PSCs protect tumor cells from apoptosis, radiotherapy, and chemotherapy^{59,60}. Bachem *et al.* have demonstrated in an *in vivo* study that the increased ECM deposition in pancreatic cancers results from the paracrine stimulation of PSCs by cancer cells⁵⁰. Another *in vivo* study showed that when PSCs and Panc-1 epithelial tumor cells are subcutaneously injected in a 1:1 mix in mice, these tumors grow significantly faster and are richer in α -SMA and collagen than subcutaneous tumors consisting solely of Panc-1 cells⁶¹. Very recently, autophagy of pancreatic stellate cells induced by environmental stress and cell-stroma interactions, was reported to be associated with histological grading, peritoneal dissemination, perivascular invasion and lymph node metastasis⁶².

1.8 Tumor stroma contributes to drug resistance and presents a barrier for therapeutics

PDAC has a high resistance to chemotherapeutics. There are multiple reasons for this chemo resistance including: (i) reduced drug uptake, (ii) increased energy-dependent drug efflux and (iii) cellular alteration that effect drug

cytotoxicity such as reduced apoptosis and a dysregulated drug metabolism⁶³. For example, a novel molecular mechanism has been proposed for TGF- β -activated PSCs which express cysteine-rich angiogenic inducer61 (CYR61), a matricellular protein regulating the nucleoside transporters hENT1 and hCNT3 responsible for the cellular uptake of gemcitabine⁶⁴.

Another cause of PDAC chemo resistance involving PSCs is the extensive PSC-induced desmoplastic reaction in PDAC, resulting in intra-tumoral hypoxia and a self-perpetuating fibrosis cycle⁶³. Activated PSCs additionally contribute to the chemoresistance via the hypoxia-fibrosis cycle⁶³. Thereby, drug delivery to cancer cells is impaired while the epithelial to mesenchymal transition (EMT) and genetic instability of these cells is induced⁶⁵⁻⁶⁷.

In general, stroma-rich tumors such as pancreatic cancer are characterized by an abundant amount of ECM proteins, produced to a high extent by PSCs, which act as a physical barrier and therefore prevent drug penetration through blood vessel constriction^{42,68-70}. Within PDAC tumor, solid stress has been closely related to drug resistance in PDAC and therapeutic strategies decreasing solid stress show potential therapeutic benefit^{71,72}. ECM components such as collagen and hyaluronic acid and PSCs are the main components of the stroma causing solid stress⁴⁸. A few studies investigating the effect of the stroma and/or its' components on drug penetration have been performed. Inhibiting hedgehog signaling to deplete tumor stromal tissue could enhance the delivery of chemotherapy in PDAC tumor-bearing mice⁶⁸. Other studies have enzymatically degraded hyaluronic acid in the tumor stroma which resulted in normalized interstitial fluid pressure, re-expansion of the vasculature, increased tumor suppression with gemcitabine and prolonged survival time^{69,70}.

1.9 Therapeutic strategies to modulate the tumor stroma

Since the contribution of the tumor stroma to the progression of PDAC has been highlighted, a number of strategies have been under intense investigation to disrupt or modulate the tumor stroma. The hedgehog pathway is known to support the tumor stroma via paracrine signaling from neoplastic to stromal cells⁶⁸. Therefore, Olive *et al.*⁶⁸ investigated the effects of a stroma depleting hedgehog inhibitor IPI-926 on the delivery and efficacy of gemcitabine. This combination therapy increased the intra-tumoral vascular density as well as the intra-tumoral concentration of gemcitabine, resulting in a transient stabilization

of the disease ⁶⁸. However, a follow up phase II clinical trial with IPI-926 was discontinued due to increased mortality ⁷³. Similar to IPI-926, other stroma-depleting therapeutic strategies did not improve patient survival and in some cases were associated with toxicity. Özdemir *et al.* performed a study in which α -SMA expressing myofibroblasts were depleted in transgenic mice, resulting in invasive, undifferentiated tumors with enhanced hypoxia, epithelial-to-mesenchymal transition, cancer stem cells and reduced survival ⁷⁴. Additionally, Rhim *et al.* ⁷⁵ deleted sonic hedgehog in a mouse model of PDAC, resulting in tumors with reduced stroma content. These tumors were more aggressive, exhibited undifferentiated histology, increased vascularity and heightened proliferation compared to controls ⁷⁵.

On the one hand, these findings brought up the theory that the stroma has tumor suppressive properties. On the other hand, the negative outcome of stroma depleting studies might be due to the complete removal of fibrotic barriers which hold tumor cells in place and prevents their metastasis. Therefore, modulating the tumor stroma to dampen its tumor promoting activities rather than depleting the stroma could result in therapeutic benefits ³⁸. More recently, the benefits of this strategy have been demonstrated by us and others.

The PEGylated hyaluronidase (PEGPH20) has been assessed in combination with gemcitabine, improving survival and attenuating tumor growth in mice when compared with gemcitabine alone ⁶⁹. In a phase Ib study, PEGPH20 in combination with gemcitabine showed an increase in progression-free and overall survival rates of patients with metastatic PDAC, but also thromboembolic event in 29 % of patients ⁷⁶. PEGPH20 is currently tested in advanced clinical trials.

Inhibition of MMPs is another therapeutic strategy for the treatment of PDAC. Marimastat, a multifamily MMP inhibitor, was assessed in a randomized clinical trial in which the 1-year survival rate of patients treated with marimastat was similar to those treated with gemcitabine ⁷⁷. However, when marimastat was tested as a combination therapy with gemcitabine, it showed no additional benefits compared to gemcitabine ⁷⁸. Bay 12-9566, an inhibitor of MMP-3, -9 and -13, has also been compared to gemcitabine in a phase III clinical trial but showed less therapeutic efficacy in advanced PDAC ⁷⁹.

Connective tissue growth factor, which is known to induce PSC proliferation, migration and ECM production, is another potential therapeutic target ⁸⁰. CTGF has been blocked using the monoclonal antibody FG-3019 ⁸¹ or antagonist,

blocking the interaction between CTGF and chemokine receptors ⁸². FG-3019 induced the effectiveness of gemcitabine while not affecting intra-tumoral accumulation of gemcitabine in a mouse model of PDAC ⁸¹.

Pirfenidone, an anti-fibrotic agent, has been shown to reduce PSC proliferation, invasion, migration, secretion of collagen and periostin and decreased overall tumor growth, peritoneal disseminated nodules and liver metastasis in an orthotopic mouse model induced with PSCs and cancer cells ⁸³. When pirfenidone treatment was combined with gemcitabine, tumor growth was attenuated compared to gemcitabine alone ⁸³. Additionally, pirfenidone has been used in combination with N-acetyl cysteine and reduced desmoplasia in an orthotopic hamster model, induced with HapT1 pancreatic cancer cells ⁸⁴.

Two different inhibitors have been used against angiotensin II, which stimulates proliferation of PSCs through the protein kinase C and EGF-ERK pathway ⁵³. Olmesartan is an angiotensin II type I receptor blocker, which decreased the proliferation and collagen I synthesis of PSCs and inhibited the growth and α -SMA expression in subcutaneous tumors consisting of AsPc-1 and PSCs ⁸⁵. Another angiotensin II type I receptor inhibitor, losartan, reduced stress in solid tumors, resulting in increased vascular perfusion which enhanced chemotherapy efficiency in pancreatic and breast cancer models ⁸⁶.

Other strategies focus on reprogramming activated PSCs into their quiescent state to diminish PSC-induced tumor promotion ⁵³. When activated, PSCs lose their cytoplasmic vitamin A (retinol) storing lipid droplets. Patients with PDAC are often deficient in vitamin A and D analogs, which supports the activation of PSCs ^{45,87}. Treatment of activated PSC *in vitro* with all-trans retinoic acid (ATRA) showed inhibitory effects on PSC migration and collagen synthesis ⁸⁸. Additionally, ATRA treatment of PSCs induced quiescence in PSCs, leading to a reduction in their proliferation and increased apoptosis of surrounding cancer cells ⁸⁹. Currently, a phase IB study is underway investigating ATRA along with gemcitabine and nab-paclitaxel in PDAC ⁹⁰. Additionally, the vitamin D receptor (VDR) has been shown to be a master transcriptional regulator to regain the quiescent state of PSCs ⁹¹. Calcipotriol, a VDR ligand in combination with gemcitabine, could induce stromal reprogramming in KPC mice, increase drug accumulation in tumors, reduce tumor volume and increase survival compared to gemcitabine treatment alone ⁹¹.

Another interesting class of targets to reprogram PSCs are microRNAs (miRNAs), small non protein coding single stranded RNA molecules, which

regulate posttranscriptional gene expression³⁵. A single miRNA sequence can regulate hundreds of target genes and can thereby miRNAs act as oncogenes or tumor suppressors³⁵. Therefore, blocking of oncogenic miRNAs with antisense RNA strands shows therapeutic potential³⁵. MicroRNA-21 has been observed to be upregulated in CAFs of PDAC and was associated with poor survival⁹². Although the function of miRNA-21 in the stroma has not been understood, it has shown to reduce the growth of MiaPaCa-2 tumors in mice⁹³. In TGF- β -activated PSCs and PDAC biopsies, miRNA-29a and miRNA-29b were found to be decreased⁵³. Restoration of miR-29 expression in PSCs reduced stroma accumulation and tumor growth⁶⁶. In our lab, miRNA-199a and miRNA-214 have been identified to be overexpressed in CAFs and activated PSCs⁹⁴. Additionally, their role in PSC differentiation, migration, tube formation by endothelial cells, PSC-induced paracrine effects on tumor cells and growth of 3D-heterospheroids composed of PSCs and cancer cells has been demonstrated⁹⁴.

Moreover, we have recently found that the endogenous lipid lipoxin A4 (LXA4) is capable to attenuate the activation of human PSCs into CAF-like myofibroblasts *in vitro* and to reduce fibrosis and tumor growth of stroma-rich subcutaneous tumors *in vivo*⁹⁵.

1.10 Targeting strategies for drug delivery to the tumor stroma

The targeted delivery of potential therapeutic agents into their target cells has the potential to enhance the therapeutic efficacy of drugs and at the same time reduce off-target effects. In general, targeting strategies include direct modification of a drug or modification of a drug delivery system (e.g. liposomes or polymeric nanoparticles), which minimize toxicity, increase bioavailability and/or improve the pharmacology of a drug⁹⁶. There are two different strategies for drug targeting: active and passive targeting. Passive targeting is based on the accumulation of drugs at the target site based on the blood flow, pathophysiological characteristics of the organ and physicochemical characteristics of the drug⁹⁷. To achieve passive targeting, drug or drug delivery systems need to have a prolonged circulation time which can only be achieved if they can escape clearance by the reticuloendothelial system (RES)⁹⁷. Therefore, drug delivery systems and imaging applications are often modified with polyethylene glycol (PEG) at their surface, which results in reduced uptake by the RES, an increased circulation time and can prevent the aggregation and

association with serum proteins^{97,98}. These modifications allow drug delivery systems to reach their target site via fenestration in leaky and highly permeable blood vessels with poor lymphatic drainage, which are commonly present in rapidly expanding solid tumors⁹⁷. The described phenomenon is commonly known as the enhanced permeability and retention (EPR) effect and applicable for nanoparticles or macromolecules with a hydrodynamic size below 200 nm⁹⁹⁻¹⁰¹. Active targeting is based on the interaction of a ligand on the drug or drug delivery system and its respective targeting receptor or biomolecule on the surface of a target cell⁹⁷. By this mechanism, the ligand modified drug or drug carrier associates and is taken up into the target cell. Active targeting can additionally be achieved by the physicochemical characteristics of the drug, including size, shape and charge, which can cause increased tissue penetration and cellular uptake^{97,99}.

The pathogenic structure of the tumor microenvironment negatively affects the major and most important routes of molecular transport, which are highly efficient in healthy organs such as vascular, trans-vascular and interstitial transport and cellular uptake, preventing drug penetration and efficacy in the tumor and hence drug delivery to CAFs or activated pancreatic stellate cells⁹⁷.

The challenges to achieve drug delivery to activated PSCs in the tumor stroma include overcoming the barrier of morphologically abnormal blood vessel basement membrane in the tumor vasculature¹⁰² and infiltrated immune cells with a high phagocytic capacity in the vascular area¹⁰³. To overcome these barriers and improve drug delivery to the tumor stroma, strategies as solid stress attenuation, improvement of the tumor vasculature, reduction in the interstitial fluid pressure and improvement of the physicochemical characteristics of therapeutic agents have been applied^{104,105}. However, specific and active drug delivery to CAFs or activated PSCs in the complex and heterogeneous tumor stroma is still evolving.

For example, Bansal *et al.* have synthesized a PDGFR- β specific interferon gamma (IFN γ) construct (PPB-HAS- IFN γ) which delivered IFN γ to fibroblasts and pericytes and inhibited angiogenesis and tumor growth in melanoma model. In a very recent study, doxorubicin carrying cell penetrating peptide nanoparticles were modified with a monoclonal antibody against fibroblast activation protein- α (FAP- α), expressed on CAFs, and increased the cellular uptake and efficacy of doxorubicin⁹⁶. In a Pan02 mouse model for pancreatic cancer a conjugate of docetaxel, PEG and acetylated carboxymethylcellulose,

accumulated in CAFs, reduced metastatic potential and increased the survival of Pan02 bearing mice ¹⁰⁶.

1.11 Aim of the thesis

The aim of this thesis is to identify novel therapeutic targets and potential therapeutics to dampen the tumor promoting functions of PSCs in PDAC. Additionally, we focus on the identification of targets for drug delivery and imaging and the design of targeting ligands for this purpose.

To achieve this, we aimed to:

- develop novel cell penetrating peptide-based nanoparticles for the specific delivery of miRNA to human PSCs (hPSC) to inhibit their activation
- investigate the effect of the bioactive lipid, lipoxin A4 (LXA4), on hPSC activation, tumor growth and ECM deposition
- investigate the role of integrin alpha 11 (ITGA11) as a therapeutic target in hPSCs
- design a novel peptide to target ITGA11 (AXI) and study its binding affinity and specificity to ITGA11 on hPSCs and potential for targeted drug delivery to ITGA11 expressing hPSCs

1.12 Outline of the thesis

Chapter 2 highlights dysregulated miRNAs in the cell types present in the tumor stroma and their functions in the regulation of the multifaceted tumor stroma. We introduce how the expression of miRNAs can be controlled via the administration of miRNA inhibitors (antagomiR) or miRNA agonists (miR mimics). Additionally, we discuss various miRNA delivery approaches to deliver miRNA inhibitors or agonists, with therapeutic potential, into stromal cells.

In **chapter 3**, we describe how we targeted anti-miRNA-oligonucleotides (AMOs) to hPSCs to block their tumor promoting effects using a novel cell penetrating peptide (CPP)-based targeting approach, in which CPPs form nanocomplexes (NCs) with anti-miRNA-oligonucleotides. When we compared the transfection efficiency of these NCs in hPSCs, normal human fibroblasts and different human pancreatic tumor cells (Panc-1, MiaPaCa-2 and AsPc-1), we found that our NCs preferentially internalized into hPSCs. Next, we investigated the uptake mechanisms responsible for the preferential uptake of our NCs by

hPSCs. Finally, the inhibitory effects of anti-miR-199a on hPSCs and hPSC paracrine effect on tumor cells was demonstrated in 2D and 3D cell cultures.

Chapter 4 shows how we investigated the ability of LXA4 to reprogram the tumor stroma. We demonstrated that the LXA4 specific receptor FPR2/ALX is overexpressed in activated hPSCs. Next, hPSCs were treated with LXA4, which inhibited their activation, migration and PSC-induced paracrine effect on pancreatic cancer cells. The inhibitory effects of LXA4 on growth and extracellular matrix (ECM) deposition were shown in tumor-mimicking stroma-rich 3D-heterospheroids. Eventually, LXA4 demonstrated therapeutic efficacy *in vivo*, in a stroma-rich co-injection tumor model, in which LXA4 treatment reduced tumor growth and intra-tumoral ECM deposition.

Chapter 5 provides a general introduction of integrins and integrin-mediated signaling, an overview of integrin expression in fibrosis-related cell types and the interaction between integrins and growth factor receptors. Next, the role of integrins in wound healing, fibrosis-driven tumors, tumor metastasis and fibrosis is described. Finally, we focus on novel therapies based on integrin inhibition, including clinical developments, and drug delivery strategies to target integrins in the context of wound healing, fibrosis and tumor stroma.

In **chapter 6**, we describe the very first examination of the global and cellular expression of ITGA11 in human samples of PDAC. We found that ITGA11 is mostly expressed on α -SMA positive cells (CAFs and activated hPSC) in human PDAC. *In vitro*, we have shown the expression of integrin α 11 on activated hPSCs and demonstrated the significance of ITGA11 in TGF- β and Panc-1 conditioned medium mediated hPSC activation, cell growth, migration and hPSC-induced paracrine effects on Panc-1 tumor cell migration and invasion.

In **chapter 7**, we depict the staining of human PDAC and various other human organs for ITGA11 and introduce ITGA11 as a drug delivery target, based on its specific expression in the tumor stroma of PDAC. Next, we designed a novel targeting peptide (AXI) specifically binding to recombinant ITGA11 or ITGA11 on hPSCs *in vitro*. We then use AXI for the surface-modification of liposomes which facilitates their delivery into ITGA11 expressing hPSCs *in vitro*. In stroma-rich subcutaneous tumors in mice, AXI-modified liposomes showed increased tumor accumulation, penetration depth and co-localization with ITGA11 expressing cells.

In **chapter 7**, we summarize the finding of the research presented in this dissertation and discuss how these results can contribute to the development of targeted strategies to reprogram or modulate the tumor stroma of PDAC.

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Chapter 2 - MicroRNA targeting to modulate tumor microenvironment

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Abstract

Communication between stromal cells and tumor cells initiates tumor growth, angiogenesis, invasion, and metastasis. Stromal cells include cancer-associated fibroblasts, tumor-associated macrophages, pericytes, endothelial cells and infiltrating immune cells. MicroRNAs (miRNAs) in the tumor microenvironment have emerged as key players involved in the development of cancer and its progression. miRNAs are small endogenous non-protein-coding RNAs that negatively regulate the expression of multiple target genes at posttranscriptional level and thereby control many cellular processes. In this review, we provide a comprehensive overview of miRNAs dysregulated in different stromal cells and their impact on the regulation of intercellular crosstalk in the tumor microenvironment. We also discuss the therapeutic significance potential of miRNAs to modulate the tumor microenvironment. Since miRNA delivery is quite challenging and the biggest hurdle for clinical translation of miRNA therapeutics, we review various non-viral miRNA delivery systems that can potentially be used for targeting miRNA to stromal cells within the tumor microenvironment.

2.1 Introduction

The tumor microenvironment is composed of cancer cells and non-cancerous cells so-called stromal cells such as cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), pericytes, endothelial cells and infiltrating immune cells^{1,2}. Over the last decade, it was well established that stromal cells promote tumor growth, angiogenesis, invasion, and metastasis^{3,4}. These effects are observed in breast, pancreatic, liver, brain, ovarian and prostate cancer. Evidence suggests that tumor cells recruit stromal cells by secreting chemokines and growth factors, which educate them to create a tumor-favoring microenvironment⁴. The “educated” stromal cells such as CAFs, endothelial cells, pericytes, TAMs and other immune cells, interact with tumor cells as well as among themselves to stimulate tumor growth, metastasis and development of resistance to chemotherapy^{3,4}. Intervening into these interactions within the tumor microenvironment is an interesting strategy to develop novel therapies for cancer treatment.

MicroRNAs (miRNAs) are represented as a novel class of therapeutics, regulating multiple signaling pathways within the tumor microenvironment⁵. miRNAs, a class of small (17-25nt) endogenous non-coding RNAs, regulate gene expression at the posttranscriptional level and thereby control cellular processes such as differentiation, proliferation and migration⁶. miRNAs have the ability to regulate not only one but hundreds of target genes simultaneously and thereby control multiple signaling pathways⁷. Gene silencing occurs through imperfect/perfect complementary base pairing between a miRNA guide strand and the 3'UTR region of the mRNA, which leads to translational repression or mRNA degradation^{6,8}. During cancer initiation and progression, the expression levels of multiple miRNAs are aberrantly up- or down-regulated, resulting in an imbalance of cellular pathways ultimately leading to the attainment of a pathological state. In this article, we highlight dysregulated miRNAs in different tumor stromal cells and their functions in the regulation of the multifaceted tumor microenvironment. The expression of miRNAs can be controlled by administering either miRNA inhibitors (antagomiR) or miRNA agonists (miR mimics). We summarize various miRNA delivery approaches that have been or can potentially be applied to deliver miRNA as therapeutics into stromal cells.

2.1.1 MicroRNA in the tumor microenvironment

In recent years, many miRNAs have been identified in different stromal cells of the tumor microenvironment as illustrated in **Figure 1**. The **table 1** summarizes

the miRNAs in the stromal cells from different cancer types with genes regulated by these miRNAs and their functions.

2.1.2 Cancer-associated fibroblasts (CAFs)

CAFs are one of the most abundant cell types in the tumor microenvironment of many solid tumors^{9,10}. In response to inflammatory stimuli, quiescent fibroblasts differentiate into activated myofibroblast (CAFs), expressing increased levels of α -smooth muscle actin (α -SMA). CAFs secrete numerous cytokines, chemokines, and ECM components which actively participate in tumor progression, invasion and metastasis¹¹⁻¹⁴. Differentiation of fibroblasts into a CAF phenotype has been proposed to be regulated at the post-transcriptional level by miRNAs¹⁵.

Several studies have reported the importance of specific miRNAs in the activation and transdifferentiation of fibroblasts to CAFs and CAF-induced tumorigenic actions¹⁶⁻¹⁹. miRNA-21 is one of the most common miRNAs that is reported to be induced in tumor cells and CAFs of pancreatic and colorectal tumors¹⁹⁻²³. Inhibition of miR-21 using antagomiR reduced the migration/invasion of CAFs²⁰. Not only upregulation but also downregulation of certain miRNAs can induce a CAF phenotype. Tang *et al.*, identified downregulated miR-200 as a direct regulator of reprogramming fibroblasts into CAFs²⁴. Downregulation of miR-200 in normal fibroblasts accelerated their migration and invasion potential similar to CAFs²⁴. Bronisz and coworkers identified miR-320 as a downstream regulator of the PTEN (Phosphatase and tensin homolog deleted on chromosome 10) gene that controls cell proliferation and migration in CAFs and was co-expressed in tumor stroma of breast tissue. Loss of PTEN and miR-320 has shown to be involved in the reprogramming of the tumor microenvironment to promote tumor invasion and angiogenesis²⁵. Mitra *et al.*, found downregulation of miR-31 and miR-214, and upregulation of miR-155 in their miRNA profiling and reversal of the activities of these miRNA in these patient-derived CAFs reversed their phenotype¹⁸. This study suggested that miRNA reprograms fibroblasts into CAFs and, therefore, targeting of miRNA in stromal cells could be a therapeutic approach to treat cancer¹⁸. In other studies, miR-31 and miR-148a were shown to be downregulated in endometrial CAFs^{16,17}. Overexpression of miR-31 or miR-148a in these CAFs impaired their ability to stimulate migration and invasion of endometrial cancer cells, which were linked to the direct targets SATB2 and WNT10B for miR-31 and miR-148a, respectively^{16,17}.

Figure 1. miRNAs mediating changes in tumor microenvironment components. Up and down regulated miRNAs are enlisted. *Mark denotes that the specific miRNA is expressed differentially in different CAFs.

Using miRNA microarray analysis, several dysregulated miRNAs have been identified in breast CAFs e.g. miR-31-3p, 221-5p and 221-3p were upregulated and miR-205, miR-200b, miR-200c, miR-141, miR-101, miR-342-3p, let-7g, and miR-26b were downregulated^{26,27}. Furthermore, many miRNA-responsive target genes and signaling pathways were revealed; that regulate different cellular processes such as cell differentiation, adhesion, migration, proliferation, and cell-cell interaction²⁶. It is important to note that miR-31 was found to be downregulated in CAFs derived from ovarian and endometrial tumors^{17,18} while it was upregulated in CAFs from breast tumor²⁶, indicating that the same miRNA can have dual activities, thereby acting as an oncogene in one tissue and as a tumor suppressor in another.

In prostate CAFs, miR-15a and miR-16 were shown to be downregulated in CAFs obtained from 23 patients²⁸. Downregulation of miR-15 and miR-16 in CAFs promoted tumor progression through the reduced post-transcriptional repression of Fgf-2 and its receptor Fgfr1. These pathways act on both stromal and tumor cells to enhance cancer cell survival, proliferation and migration²⁸. Reconstitution of tumor-suppressive miR-15a and miR-16 in CAFs inhibited tumor-promoting ability of stromal cells as shown in a co-injection (tumor cells and CAFs) mouse model, proposing these miRNA as potential targets for the development of novel therapies²⁸.

Additionally, Li *et al.*, demonstrated that miR-149 mediates the crosstalk between the tumor cell and CAFs in gastric cancer via prostaglandin E2 (PGE2) and interleukin (IL)-6 signaling pathways²⁹. While it remains unclear how PGE-2 modulates this crosstalk, it was demonstrated that by targeting IL-6, miR-149 inhibited fibroblast activation²⁹. The effects of CAFs on gastric cancer development were negatively regulated by miR-149²⁹. Additionally, CAFs enhanced the epithelial to mesenchymal transition (EMT) and stem-like properties of gastric cancer cells in a miR-149/IL-6 dependent manner²⁹. In another study, miR-106b have been identified as a marker of poor prognosis in gastric cancer³⁰. Downregulation of miR-106b expression in CAFs resulted in significantly inhibited CAF-induced gastric cancer cell migration and invasion mediated through PTEN pathway³⁰. These studies reveal the miRNA targets as diagnostic biomarkers and therapeutic targets for developing the anti-CAF therapy.

2.1.3 Tumor-associated macrophages and immune cells:

Macrophages and other immune cells such as T-cells, natural killer (NK) cells are the major inflammatory cells infiltrating into the tumor microenvironment^{3,31}. In the past, the infiltration of innate and adaptive immune cells into the tumor microenvironment was considered as an immune attack against cancer³¹. However, now it is widely accepted that immune cells do also promote cancer initiation, progression, and metastasis³². Macrophages within the tumor microenvironment can be polarized from anti-tumorigenic M1 macrophages to pro-tumorigenic M2 macrophages (Tumor-associated macrophages (TAMs), via changes in their metabolic pathways and the production of cytokines (CSF-1, IL-4, IL-13) by immune cells³¹. TAMs promote tumor progression by stimulating angiogenesis, tumor cell migration and extravasation at metastatic sites and suppressing antitumor immunity thereby reduce patient survival^{31,33,34}. Recent studies have unraveled the significance/dysregulation of miRNA in

macrophages³⁵. In a study by Graff *et al.*, miRNA expression profiles were determined in monocyte-derived macrophages differentially polarized into M1, M2a, M2b and M2c phenotypes³⁶. They reveal several miRNAs to be uniquely regulated in human macrophages polarized into M1 (miR-125a-3p, miR-26a-2*), M2a (miR-193b) and M2b (miR-27a*, miR-29-b-1*, miR-132, miR-222*)³⁶. Herein we report approaches through which dysregulated miRNAs have been targeted to reprogram miRNA expression in TAMs and thereby suppress their pro-tumorigenic properties.

Squadrito *et al.*, showed that miR-511-3p, encoding for the macrophage mannose receptor, is upregulated in MRC1⁺ TAMs^{37,38}. Enforcing miR-511-3p expression in MRC1⁺ TAMs resulted in the suppression of protumoral genes and inhibited tumor growth with a change in blood vessel morphology. These effects were attributed to ROCK2, a direct target of miR-511-3p³⁷. The protein expression of transcription factor C/EBP β showed elevated levels in TAMs as well as human hepatocellular carcinoma tumor sections *in situ*³⁹. C/EBP β expression was correlated with the production of cytokines in tumor-activated monocytes and shown to be regulated by sustained reduction of miR-155³⁹. Overexpression of miRNA-155 was shown to attenuate the production of the cytokines (IL-6, IL-10, TNF- α) by suppressing C/EBP β expression which led to inversion of protumoral M2 into pro-inflammatory M1 macrophages, as demonstrated by upregulated M1 markers (TNF- α , NOS2, and IL-12) and downregulated M2 markers (Arg1, Ym1, Msr2, Fizz1, and IL-10)⁴⁰. More recently, ectopic expression of miR-26 in hepatocellular carcinoma (HCC) cells suppressed the tumor growth, downregulated the expression of macrophage colony stimulating factor (M-CSF) through the PI3K/Akt pathway, and suppressed the infiltration of macrophages in tumors⁴¹. In addition, miR-26a expression was inversely correlated with M-CSF expression and the infiltration of macrophages into the tumor tissue of HCC patients⁴¹.

Besides TAMs, other immune cells such as myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells and T cells also express miRNAs that regulate their pro-tumorigenic potential. MDSCs negatively regulate immune responses by suppressing the anti-tumor functions of CD4⁺ and CD8⁺ T cells by inhibiting the activities of natural killer cells⁴². miR-155 and miR-21 are reported as the most upregulated miRNAs in MDSCs from bone marrow cells, regulating PTEN and SHIP1, respectively⁴³. They promote STAT3 activity inducing MDSC expansion that promote tumor aggressiveness via immunosuppression⁴³. In HCC, positive for the hepatitis B virus, suppressed

levels of miR-34a resulted in the enhanced production of chemokine CCL22, thereby recruiting regulatory T cells (Tregs) into the tumor microenvironment to facilitate immune escape⁴⁴. In human melanoma upregulation of miR-30b/-30d correlates with stage, metastatic potential, shorter time to recurrence and reduced overall survival⁴⁵. Upregulation of miR-30d in the immunocompetent mice triggered immunosuppressive properties at the lung metastatic site, shown by an enhanced infiltration of Tregs⁴⁵. Bezman *et al.*, suggested that miR-150 differentially controls the development of natural killer (NK) and invariant NK T (iNKT) cells by targeting c-Myb. Mice with miR-150 deletion showed a defect in their ability to develop mature NK cells⁴⁶. Overexpression of miR-150 promotes the development of mature NK-cells, which were highly responsive to activation⁴⁶. Contrarily, the number of iNKT cells was reduced upon miR-150 upregulation⁴⁶. MiR-155 was found to regulate partly interferon-gamma production in human NK-cells by downregulating SHIP1, making it a potential target in neoplastic disease⁴⁷.

2.1.4 Tumor vascular cells

Endothelial cells together with pericytes are the major cellular components of tumor blood vessels, thus playing an important role in angiogenesis during tumor development²⁻⁴. Chan *et al.*, identified avian erythroblastosis virus E26 (v-ets) oncogene homolog-1 (Ets-1), an angiogenesis-related transcription factor, regulated by miR-200b⁴⁸. Ectopic expression of miR-200b reduced the tube formation and cell migration ability of human microvascular endothelial cells (HMECs) *in vitro* by targeting Ets-1 and its associated genes, matrix metalloproteinase-1 and VEGF receptor-2⁴⁸. Interestingly, the authors demonstrated that miR-200b downregulation is hypoxia-induced and represses Ets-1 expression to promote angiogenesis in HMECs⁴⁸. Hypoxia stimulation also influences several miRNA expression levels in rat cortical pericytes compared to normoxic conditions⁴⁹. Real-time PCR data revealed changes in the expression of miRNAs associated with hypoxia-inducible factor-1 α (HIF-1 α) (miR-322, miR-199a), TGF- β 1 (miR-140, miR145, miR-376b-3p) and VEGF (miR-126a, miR-297, miR-16 miR-17-5p)⁴⁹.

In human umbilical vein endothelial cells (HUVECs), miR-29c was identified to regulate cell cycle, proliferation, and angiogenesis *in vitro*, likely mediated by suppressing Insulin-like growth factor-1⁵⁰. miRNA-7 was identified as a negative regulator of angiogenesis, strongly reducing cell viability, tube formation, sprouting and migration *in vitro*⁵¹. In an *in vivo* murine neuroblastoma tumor model, angiogenesis and tumor growth were significantly inhibited upon

local administration of miR-7⁵¹. MiR-155, which is known to be upregulated in various human cancers, is also involved in the angiogenesis of breast cancer by targeting the von Hippel-Lindau (VHL) tumor suppressor gene⁵². Mammary fat pad xenotransplantation of ectopically expressed miR-155 strongly induced angiogenesis, proliferation, tumor necrosis and recruitment of pro-inflammatory TAMs⁵². Moreover, miR-155 was identified as a marker for late-stage, lymph node metastasis and poor prognosis in breast cancer⁵².

Recently, miRNA array profiling revealed that endothelial cells communicate with pericytes via miRNA-containing exosomes which increased VEGF-B expression in pericytes at both gene and proteins levels⁵³. In addition, Lim et al showed that CXCL12-specific miRNAs are transported from bone marrow stroma to breast cancer cells via gap junctions and reduced CXCL12 levels as well as proliferation⁵⁴. Furthermore, mechanisms for the transfer of siRNA and miRNA through the gap junctions have been reviewed⁵⁵.

2.2 MicroRNA delivery to the tumor stromal cells

To turn miRNAs into therapeutics, it is essential to deliver antagomiRs or miRNA mimics cell specifically into the target cells. However, naked miRNAs are unable to pass through cell membrane due to their hydrophilicity, polyanionic nature, and high molecular weight. Intravenous administration of naked miRNA leads to poor tissue distribution due to rapid renal excretion (plasma half-life~1 hour) and degradation by serum RNases⁵⁶. *In vivo* application of miRNA either requires a chemical modification or formulation into delivery systems. There are several articles describing targeting strategies for miRNA delivery⁵⁷⁻⁶⁴. Despite having many strategies reported, the number of successful delivery approaches *in vivo* is still limited.

2.2.1 Strategies for miRNA delivery

Encapsulation of miRNAs into nanoparticles has been addressed to protect miRNAs from degradation by nucleases, resulting in an improved circulating half-life when administered systemically⁶⁵. Viral and non-viral encapsulation strategies have been investigated for this purpose. Viral-based delivery of nucleotides shows high transfection efficiencies, but will not be discussed in this review due to concerns regarding strong inflammatory side effects⁶⁶. The advantages of non-viral delivery systems over viral delivery systems are their well-defined molecular composition, simpler manufacturing and considerably

lower immunogenicity⁶⁶. The advantages of non-viral delivery systems over viral delivery systems are their well-defined molecular composition, simpler manufacturing and considerably lower immunogenicity⁶⁷.

Non-viral delivery systems that have been investigated for their usability as miRNA carrier systems are liposomes, lipoplexes, and polyplexes. Liposomes are composed of phospholipids possessing a hydrophilic head linked to a hydrophobic tail⁶⁸. This amphiphilic structure allows them to form vesicles with an inner aqueous compartment in which miRNAs can be encapsulated⁶⁸. Cationic lipids have been used to form complexes with negatively charged miRNA, called lipoplexes⁶⁹. Liposomes and lipoplexes have shown to protect miRNAs from degradation by nucleases, resulting in an improved circulating half-life when administered systemically⁶⁵. In addition, cationic polymers that are frequently used for intracellular delivery are polyethyleneimine (PEI) and polyamide amine dendrimers (PAMAM). PEI- and PAMAM-based polyplexes have been used successfully for the delivery of miRNA and *in vitro* and *in vivo*^{70,71}. Systemic delivery of PAMAM had toxic effects on the liver and the kidney in mice⁷². For PEI, clinical translation has been hampered by dose-dependent toxicity upon systemic administration⁷³. Similar toxicity issues can be expected from lipoplexes.

2.2.2 MicroRNA delivery to tumor stroma cells

The number of studies for the delivery of miRNAs to the compartments of the tumor stroma *in vivo* is highly limited. Polyplexes based on polyethyleneimine (PEI) delivered dicer substrate RNA duplexes, mimicking the structure of endogenous precursor miRNA-155 hairpin (Dmi155), into ID8-Defb29/Vegf-A tumors in mice⁷⁴. Increasing the levels of miRNA-155 in tumor-associated dendritic cells silenced multiple immunosuppressive mediators⁷⁴. These changes lead to the transformation of tumor infiltrating dendritic cells from their immunosuppressive phenotype to immunostimulatory cells⁷⁴. Post-transformation of these cells were capable of triggering antitumor responses thereby inhibiting the progression of established ovarian cancers in mice⁷⁴.

Endothelial cells are the only cell type present in the tumor microenvironment, which have been actively targeted *in vivo* for miRNA delivery. Anti-angiogenic effects in tumors were achieved in a study performed by Ando *et al.*, using lipoplexes modified with a PEG chain bearing the angiogenic vessel targeting peptide Ala-Pro-Arg-Pro-Gly (APRPG)⁷⁵. The APRPG-PEG-modified liposomes were used to form complexes with miR-499. The resulting lipoplexes

were injected into mice bearing Colon 26 NL-17 xenografts⁷⁵. The lipoplexes accumulated in both angiogenic vessels and cancer cells resulting in downregulation of miR-499-regulated proteins and vascular endothelial growth factor (VEGF)⁷⁵. Following an injection of 0.5 mg/kg of the lipoplexes, tumor growth in mice was significantly inhibited⁷⁵.

In a study by Liu *et al.*, anti-miR-296 antagomiR was delivered into human vein umbilical vein endothelial cells⁷⁶. PEGylated liposome-polycation-hyaluronic acid (LPH) nanoparticles conjugated with a cyclic RGD peptide (cRGD) were used as carrier systems for the specific targeting of $\alpha_v\beta_3$, a receptor present on endothelial cells in the tumor neovasculature⁷⁶. They reported inhibition of tube formation and endothelial cell migration, and the significant upregulation of hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), one of the genes suppressed by miRNA-296⁷⁶. A matrigel plug assay was performed to analyze the effect of anti-miR-296 delivery on *in vivo* angiogenesis⁷⁶. As a result, a decrease in microvessel formation by preventing CD31-positive endothelial cells from invading into the matrigel in combination with an induction of HGS in angiogenic endothelial cells was observed⁷⁶.

Anand *et al.*, aimed to repress neovascularization in tumors by inhibiting miRNA-132 levels via the delivery of anti-miRNA-132⁷⁷. Liposomal nanoparticles composed of distearoylphosphatidylcholine (DSPC), cholesterol, dioleoylphosphatidylethanolamine (DOPE), distearoylphosphatidylethanolamine (DSPE)-mPEG2000 modified with a DSPE-cyclic RGD peptide, targeting $\alpha_v\beta_3$ were used as a delivery system⁷⁷. miR-132, highly overexpressed in the endothelium of human tumors, mediated loss of p120RasGAP thereby inducing neo-vascularization⁷⁷. By restoring p120RasGAP levels using anti-miR-132 delivery, neovascularization was suppressed, and tumor burdens were decreased in an orthotopic xenograft mouse model of human breast carcinoma, and vessels were maintained in their non-pathological resting state⁷⁷. Systemically administered miRNA7 loaded peptide/polymer-based delivery systems modified with a cRGP ligand targeting the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ ⁷⁸, targeted human glioblastoma xenografts in mice and strongly reduced angiogenesis and tumor proliferation.

Table 1. List of miRNAs in various tumor stromal cells.

Cell type	miRNA	Cancer type	Target gene	Functions
CAFs	miR-155/214 ¹⁸	Ovarian	CCL5	Differentiation
	miR-31 ¹⁷	Endometrial	SATB2	Migration, Invasion
	miR-148a ¹⁶	Endometrial	WNT10B	Migration
	miR-15/-16 ²⁸	Prostate	FGF2	Migration, Proliferation
	miR-320 ²⁵	Breast	ETS2	Invasion, Blood vessels, Tumor growth
	miR-106b ³⁰	Gastric	PTEN	Migration, Invasion
	miR-200c ²⁴	Breast	Fli-1, TCF12	Migration, Invasion
	miR-149 ²⁹	Gastric	IL-6	Differentiation and Anti-stromal effects on tumor cells
	miR-21 ²¹	Colorectal	RECK	Differentiation
Macrophages	miR-155 ³⁹	Hepatocellular carcinoma	C/EBP β	Repolarization towards M2 phenotype
	miR-511-3p ³⁷	Macrophages Expressing MRC1	ROCK2	Tumor growth, blood vessel morphology
	miR-26a ⁴¹	Hepatocellular carcinoma	M-CSF	Recruitment of macrophages
T cells	miR-34a ⁴⁴	Hepatocellular carcinoma	CCL22	Infiltration of immune cells
	miR-30d ⁴⁵	Melanoma cells	GALNT7	Infiltration of Tregs cells
NK cells	miR-150, miR-155 ^{46,47}	NK cells	SHIP1	Activation of NK cells
Endothelial cells	miR-200b ⁴⁸		ETS1	Blood vessels, migration
	miR-29c ⁵⁰		IGF1	Angiogenesis, proliferation
	miR-7 ⁵¹	Glioblastoma		Cell viability, migration, Blood vessels
	miR-155 ⁵²	Breast	VHL	Invasion, migration, Proliferation, blood vessels

Pericytes	miR-145, miR-126a, miR-199a ⁴⁹		Fli1	Migration
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2.3 Conclusion

In recent years, miRNAs have been extensively discovered in the tumor stromal cells either to be used as biomarkers or to show their potential for inhibiting the cellular processes. Undoubtedly, the miRNA field has a high potential to develop novel therapeutics against cancer; however, at the same time development of technologies to deliver miRNAs to specific cells are highly essential to utilize them for therapeutic purposes. With this review, we bring together the fields of tumor biology and miRNA delivery, which will surely benefit both biologists and technology developers.

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Chapter 3 - Anti-microRNA targeting using peptide-based nanocomplexes to inhibit differentiation of human pancreatic stellate cells

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Abstract

Aim: To develop novel peptide-based nanocomplexes (NCs) for delivery of anti-miRNA oligonucleotides to human-derived pancreatic stellate cells (hPSCs), precursors of cancer-associated fibroblasts. **Materials & methods:** NCs of anti-miRNA oligonucleotides and cell-penetrating peptides (different variants) were formed and characterized. The effects of anti-miR-199a delivery on hPSC differentiation and 3D heterospheroid formation were investigated. **Results:** Dimeric cell-penetrating peptide based NCs (NC-2) showed 130-fold higher uptake by hPSCs compared with monomer-based NCs (NC-1) and tenfold higher uptake compared with general fibroblasts and different pancreatic tumor cells. Interestingly, delivery of anti-miR-199a inhibited hPSC differentiation into cancer-associated fibroblasts and inhibited the size of 3D heterospheroids comprised of hPSCs and tumor cells. **Conclusion:** Our NCs present a highly efficient anti-miRNA delivery system to hPSCs to inhibit their protumorigenic activity.

Graphical abstract

In this study, we developed novel cell-penetrating peptide based nanocomplexes (~40 nm) to efficiently deliver anti-miRNA-199a to primary human pancreatic stellate cells (hPSCs). Our nanocomplexes significantly inhibited the differentiation of hPSCs into tumor-promoting human pancreatic tumor stromal myofibroblasts and reduced the growth of in vitro tumor heterospheroids composed of hPSCs and Panc-1 epithelial cancer cells.

Keywords: cancer-associated fibroblasts, cell-penetrating peptides, microRNA-199a, miRNA delivery, nanocomplexes, pancreatic cancer, tumor microenvironment

3.1 Introduction

miRNAs are nonprotein-coding ssRNA molecules, ranging from 19 to 25 nucleotides, which regulate posttranscriptional mRNA expression via complementary binding to their target mRNA. By this mechanism, miRNAs possess the ability to control hundreds of target genes simultaneously and thereby regulate cellular pathways involved in cell survival, proliferation, differentiation, apoptosis and metabolism¹⁻³. In a pathological state, such as cancer, the expression levels of various miRNAs are greatly altered, contributing to the imbalance between cellular pathways and thereby attainment of the pathological state^{1,4}. By means of miRNA modulators (miRNA mimics and anti-miRNA modulators), the pathological state can be inhibited or reversed, making miRNA-based therapeutics an attractive strategy to treat a disease⁵⁻⁸. However, the poor pharmacokinetic profile of miRNA oligonucleotides as a consequence of their rapid degradation by serum nucleases, rapid renal elimination and nonspecific distribution limits their systemic applications⁵⁻⁸. Furthermore, at the cellular level, oligonucleotides are unable to penetrate cellular membranes due to their highly negative charge. Nanoparticle-based delivery systems through which miRNA oligonucleotides can be protected from degradation and delivered intracellularly are therefore under intense investigation^{5,7-10}. Various methods based on nonviral vectors, such as liposomes, micelles, polymeric nanoparticles, nanospheres, antibody and cell-penetrating peptides (CPPs), have been investigated for miRNA delivery^{1,10,11}. Especially, CPPs are powerful tools for intracellular delivery of different cargos, because of their ability to induce efficient cellular uptake and their low toxicity^{12,13}. CPPs are short peptides with a length of 8-30 amino acids¹⁴. Their positive charge enables them to spontaneously form noncovalent nanocomplexes (NCs) with negatively charged oligonucleotides via electrostatic interactions^{13,15}. After coming into contact with cells, these complexes are internalized through various mechanisms, as reviewed by Margus *et al.*¹⁶. Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive cancer types, for which, to date, no treatment has had a significant impact¹⁷.

PDA is characterized by a rapid progression, early-on metastasis formation and a high resistance to chemo- and radiotherapy¹⁸⁻²⁰. PDA possesses a dense desmoplastic reaction surrounding malignant cells, known as tumor stroma¹⁹⁻²³. A major cell type of the tumor stroma are cancer-associated fibroblasts (CAFs) which in the pancreatic tumor stroma mainly originate from resident pancreatic stellate cells (PSCs)^{21,24,25}. During carcinogenesis, PSCs become activated and

undergo subsequent functional alterations, resulting in an increased proliferation and migration potential^{22,26}. Once activated, PSCs secrete mutagenic substances, which stimulate tumor growth, invasion and resistance to chemo- and radiotherapy^{21,22}. Recent evidence suggests that both pancreatic tumor cells and stromal PSCs have dysregulated miRNA expression levels^{27,28}.

In this study, we aimed to target anti-miRNA oligonucleotides (AMOs) to inhibit miRNAs in stromal PSCs to block their protumorigenic effects. To deliver AMOs into primary human-derived PSCs (hPSCs), we designed a CPP-based targeting approach, in which CPPs form NCs with oligonucleotides. Nanoscale particles are well known to yield effective tumor delivery due to the enhanced permeability and retention effect. First, we used an arginine-rich CPP¹¹ to form NCs with AMOs and found that these NCs poorly internalized into hPSCs. We hypothesized that this lack of activity might be attributed to the unavailability of the CPP on the surface of the NCs to interact with cells. To allow for direct interaction between CPPs and cells and yet achieve nanocomplex formation with AMO, we designed a disulfide-containing dimeric form of the CPP reasoning that one CPP chain will be complexed with the AMOs and the other CPP chain will be available to interact with the cells. Additionally, the introduction of disulfide bridges into CPPs has shown to result in their cellular association and uptake via disulfide exchange and supports their endosomal escape^{29,30}. To explore the value of disulfide-mediated dimerization for the delivery of AMO, we prepared the NCs with monomeric or dimeric CPPs and compared their ability to transfect hPSCs. Furthermore, we also compared the transfection efficiency of these NCs in hPSCs, normal human fibroblasts and different human pancreatic tumor cells (Panc-1, MiaPaCa-2 and AsPc-1). We also investigated the uptake mechanisms of NCs in hPSCs and showed the inhibitory effects of anti-miR-199a in hPSCs in 2D and 3D cultures.

3.2 Methods

3.2.1 Materials

Scrambled miRNA and anti-miRNA-199a with the respective sequences: 5'CAGUACUUUUGU- GUAGUACAA'3 and 5'UGUCAUCAGACGUGUAACCAAU'3 with 2'O-methyl modification were purchased from TriLink BioTechnologies (CA, USA). FAM-labeled scrambled AMO was ordered from GenePharma (Shanghai, China). miRNAs were aliquoted in RNase-free water (Sigma Life Science, Steinheim, Germany) to a concentration of 20μM. The monomeric form of CPP (VSRRRRRRGGRRRR)

and dimeric CPP (RRRRGGRRRRRRSV-C-S-S-C-VSRRRRRRRGRRRR) were custom-synthesized from ChinaPeptides (Shanghai, China).

3.2.2 Formation of NCs

NC formation was performed by adding increasing volumes of 100 μ M monomeric or dimeric CPP solution to 15 μ l of 20 μ M miRNA. Next, milli-Q water was added to the mixture to obtain a total volume of 300 μ l. The solution was mixed properly by resuspension, followed by incubation for 1 h at room temperature (RT). By this method, NCs with charge ratios from 1:1 to 20:1 ($\pm$) were formed. Charge ratios are calculated on the basis of moles of the positively charged amino acids of the CPP and the negatively charged phosphate groups of miRNA.

3.2.3 Determination of complex size & ζ -potential

The size and zeta-potential of NCs were measured using dynamic light scattering with a Malvern Zetasizer Nano ZS (Malvern instruments, Worcestershire, UK). The complex-containing solution was mixed with 700 μ l of Milli-Q water and added to a disposable 1.5 ml cuvette (BrandTech Scientific, CT, USA) for size measurements, or injected into a folded capillary ζ -potential cuvette (BrandTech Scientific) to measure ζ -potentials.

3.2.4 Gel retardation assay

To assess the incorporation of miRNA into NCs, a gel retardation assay was performed. For each NC prepared at different charge ratios of CPP and miRNA, 60 μ l of NC solution was mixed with 20 μ l glycerol diluted 1:10 in Milli-Q water and separated on a 2% agarose gel containing 1 x Tris-borate-EDTA (TBE) buffer (Thermo Scientific, IL, USA) for 20 min at 120 V. miRNA was stained with SYBR® Gold (Invitrogen, CA, USA) according to the manufacturer's instructions. The gels were photographed in a FluorChem M imaging system (ProteinSimple, CA, USA) (excitation filter: 475 nm, emission filter: 537 nm).

3.2.5 Cells

Cells were grown in a humidified atmosphere at 37°C with 5% CO₂. Primary hPSCs were maintained in complete Stellate Cell Medium (supplemented with 2% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% Stellate Cell Growth Supplement (SteCGS; ScienCell, CA, USA). Previous to seeding hPSCs, cell culture flasks and plates were coated with L-lysine (Sigma-Aldrich, Steinheim, Germany) at a concentration of 0.0015 (w/v) in H₂O, for 1 h at 37°C and washed two-times with Milli-Q water before use. PANC-1 and MiaPaCa-2

cells were cultured in complete DMEM high glucose (4.5 g/l) with L-glutamine (GE Healthcare, Vienna, Austria) supplemented with 10% FBS (Lonza, Verviers, Belgium), 100ug/ml penicillin/streptomycin (Sigma, Zwijndrecht, The Netherlands). BJ-fibroblasts and AsPc-1–2 cells were cultured in complete RPMI medium (GE Healthcare) supplemented with 10% FBS (Lonza), 100ug/ml penicillin/ streptomycin (Sigma).

3.2.6 Cellular uptake of NCs

3.2.6.1 Fluorescence microscopy

To image cellular uptake, hPSCs, BJ-fibroblasts, Panc- 1, AsPc-1 and MiaPaCa-2 cells were seeded into an ibiTreat μ -Slide 8-well plate (Ibidi, Martinsried, Germany) at a seeding density of 1.5×10^4 cells/well, cultured as mentioned above and incubated for 18 h at 37°C. After 24 h, the culture medium was replaced with fresh serum-free medium containing NCs with a miRNA-FAM concentration of 200 nM. After 6 h of incubation, cells were washed three-times with DMEM containing 10% FBS and subsequently fixed in phosphate-buffered saline (PBS) containing 10% formaldehyde (Sigma) for 15 min. After permeabilizing the cells with 0.1 M Triton X-100 (Sigma) for 5 min, F-actin was stained with phalloidin (Life Technologies, CA, USA) at a concentration of 250 ng/ml. To stain the nuclei, a drop of mounting medium containing DAPI (Sigma) was added to each well. Cells were imaged for FAM (ex. 495/em. 520), phalloidin (ex. 540/em. 565) and DAPI (ex. 360/em. 460) using an EVOS FI fluorescent microscope (Life Technologies).

3.2.6.2 Flow cytometry

To determine cellular uptake, cells were seeded as mentioned above in 24-well plates at a concentration of 4×10^4 cells/well. For the heparan inhibition studies, cells were incubated with 2 μ g/ml heparan lithium salt (Sigma) for 30 min previous to incubation with NCs. To evaluate the uptake mechanism, cells were incubated with 10 μ M phenylarsine oxide (Sigma), 50 μ g/ml nystatin (Sigma) or 100 μ M ethylisopropyl amiloride (Sigma) in 500 μ l of PBS for 10 min prior to the transfection experiment. After 6 h of incubation with NCs containing 200 nM fluorescently labeled miRNA, cells were washed twice with PBS and detached with 50 μ l trypsin solution (Life Technologies). Trypsin was neutralized by adding 200 μ l of medium containing 2% FBS and 200 μ l of trypsin neutralizer solution (Life Technologies) to each well. The cell-containing solution was washed twice in 1ml of ice cold PBS containing 1% FBS by centrifugation and subsequently fixed in PBS containing 0.5% formaldehyde (Sigma) previous to analysis with a FACS Calibur (Becton Dickinson, NJ, USA).

3.2.7 Cell proliferation assay

To monitor the cell number, 10 μ l of alamarBlue dye (Invitrogen) in 100 μ l of Panc-1 growth media was added per well. After 4 h, the fluorescent signal was measured using a VIKTOR™ plate reader (PerkinElmer, MA, USA).

3.2.8 Quantitative real-time PCR

To investigate the effect of anti-miR-199a dimeric NCs on hPSC differentiation, hPSCs were seeded in 12-well plates (6 $\times$ 10⁴ cells/well) in complete medium. After 18 h, cells were treated with anti-miR-199a dimeric NCs for 24 h, as described previously. Thereafter, cells were activated with TGF- β 1 (5ng/ml) for 24 h. To investigate the effect of anti-miR-199a on its direct target genes, hPSCs were seeded in 12-well plates (6 $\times$ 10⁴ cells/well) in complete medium. After 18 h, cells were treated with anti-miR-199a dimeric NCs for 24 h. To study the effect of Panc-1 conditioned medium on PSC differentiation, we collected conditioned medium as described above. hPSCs were seeded in 12-well plates (6 $\times$ 10⁴ cells/well) in complete medium. After 18 h, the culture medium was replaced with fresh serum-free medium and incubated for 24 h. Next, Panc-1 conditioned medium was added to the cells for 24 h. To collect Panc-1 conditioned medium, Panc-1 were seeded in 12-well plates at a density of 15 $\times$ 10⁴ cells/ well. After 24 h, cells were washed and incubated with fresh serum-free medium for 24 h. Subsequently, the medium was collected and frozen at -20°C. Total RNA was isolated using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and the RNA amount was measured by a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). Subsequently, cDNA was synthesized with iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands). A total of 10 ng cDNA was used for each PCR reaction. The real-time PCR primers (**Table 1**) for Acta-2, Coll α 1, PDGFR- β , mTOR and RPS18 were purchased from (Sigma). Gene expression levels were normalized to the expression of the house-keeping gene RPS18.

Table 1 Sequences of forward and reverse primers used during real-time PCR

Gene	Forward Primer	Reverse Primer
RPS18	TGAGGTGGAACGTGTGATCA	CCTCTATGGGCCCCGAATCTT
α -SMA	CCCCATCTATGAGGGCTATG	CAGTGGCCATCTCATTTTCA
Collagen1 α 1	GTACTGGATTGACCCCAACC	CGCCATACTCGAACTGGAAT
PDGF β R	AGGCAAGCTGGTCAAGATCT	GCTGTTGAAGATGCTCTCCG

3.2.9 Immunocytochemistry

To stain for heparan sulfate (HS) on the surface of hPSCs and Panc-1, cells were seeded in 8-well Lab-Tek chamber slides (Thermo Fisher Scientific, NY, USA) (2×10^4 cells/well) in complete medium. After 24 h, cells were washed with PBS and subsequently fixed in PBS containing 4% formaldehyde (Sigma) for 10 min. After washing with PBS, cells were blocked with PBS containing 0.1% (w/v) bovine serum albumin (BSA; VWR, Lutterworth, UK) (PBS/BSA). Afterward, cells were incubated with anti-HS single-chain antibody HS4C3-p5118 (Matrix Biochemistry, Nijmegen, The Netherlands) diluted 1:10 in PBS/BSA for 1 h at RT. After washing with PBS, the second mouse anti-VSV monoclonal antibody p5D4 (Matrix Biochemistry) diluted 1:10 (PBS/BSA) was incubated in the cells for 1 h at RT. Next, cells were washed with PBS and incubated with rabbit antimouse polyclonal antibody (Dako, Glostrup, Denmark) diluted 1:100 in PBS/BSA for 1 h at RT. After washing with PBS, cells were incubated with Alexa Fluor 594 labeled donkey anti-rabbit (Life Technologies) diluted 1:100 in PBS/BSA for 1 h at RT. Cells were washed in PBS and nuclei stained with mounting medium containing DAPI (Sigma). Cells were imaged for Alexa 594 (ex. 540/ em. 565) and DAPI (ex. 360/em. 460) using an EVOS FI fluorescent microscope. To investigate the effect of anti-miR-199a on hPSC differentiation, hPSCs were seeded in 24-well plates (2×10^4 cells/well) in complete medium. After 18 h, cells were treated with anti-miR-199a dimeric NCs for 24 h, as described previously. After that cells were activated with TGF- β 1 (5 ng/ml) for 48 h. Next, cells were fixed and immunostained for human α -smooth muscle actin (α -SMA) and collagen1 (Col1) as described elsewhere³¹.

3.2.10 Formation of 3D heterospheroids

hPSCs were plated in 6-well plates and transfected with NC-2 NCs containing anti-miR-199a as described previously. After 24 h, cells were trypsinized and suspended in their respective culture medium at a concentration of 3×10^5 cells/ml. To evaluate cellular composition of heterospheroids, hPSCs were resuspended in medium containing 1 μ g/ml calcein red-orange AM (Life Technologies) and incubated for 1 h at 37°C. Panc-1 tumor cells were suspended to the same concentration and afterward mixed 1:1 (vol:vol) with hPSCs. One drop containing 20 μ l of cell suspension per well was pipetted into the lid of a round bottom suspension culture 96-well plate (Greiner BioOne, Alphen aan den Rijn, The Netherlands). Then the lid was inverted and placed onto a 96-well plate. Wells were filled with 150 μ l PBS for humidity. Spheroids were allowed to form for 7 days, imaged under an inverted microscope and their area was

measured using Image J software. For sectioning, 3D heterospheroids were collected, embedded in Cryomatrix (Thermo Scientific) and frozen at -80°C . Afterward, spheroid were cut into $6\text{-}\mu\text{m}$ -thick sections using a Cryotome FSE (Thermo Scientific) and mounted with fluoroshield™ with DAPI (Sigma) for staining of nuclei. Sections have been imaged using a Hamamatsu NanoZoomer Digital slide scanner 2.0HT (Hamamatsu Photonics, NJ, USA).

3.2.11 Immunohistochemical staining of 3D heterospheroids

The 3D heterospheroids were collected and cut into $6\text{-}\mu\text{m}$ -thick sections as described earlier. Cryosections were fixed in acetone at RT for 15 min and subsequently rehydrated in PBS for 10 min. Next, sections were incubated with goat-anti collagen type I (Southern BioTech, AL, USA) for 1 h at RT. Sections were washed with PBS and incubated with secondary Alexa Fluor 594 labeled donkey anti-rabbit diluted 1:100 in PBS containing 5% human serum. Sections were washed again and mounted with fluoroshield with DAPI (Sigma) for staining of nuclei. Cells were imaged for Alexa 594 (ex. 590/em. 617) and DAPI (ex. 360/em. 460) using a fluorescence microscope (Nikon Eclipse E400, Tokyo, Japan).

3.2.12 Conditioned medium

To collect conditioned medium, hPSCs were seeded in 12-well plates at a density of 6×10^4 cells/well. After 24 h, cells were transfected as described previously. After 24 h, cells were incubated with TGF- β 1 (5 ng/ ml) for 24 h. Afterwards, the cells were washed and incubated with fresh serum-free medium for 24 h. Subsequently the medium was collected and frozen at -20°C . To determine the indirect effect of hPSC-conditioned medium on Panc-1 cell growth, Panc-1 were seeded at a density of 2.5×10^3 cells/well in 96-well plates. The next day, conditioned medium was added to the cells. The cell number was monitored 0, 24 and 48 h after adding conditioned medium. Cell proliferation was determined using AlamarBlue as described previously.

3.3 Results

3.3.1 Synthesis & physicochemical properties of NCs

We prepared several NCs of negatively charged AMOs with positively charged monomeric (NC-1) or dimeric CPPs (NC-2) (**Figure 1**). Both types of NCs were prepared at various charge ratios (**Figure 2**). NC-1 and NC-2 showed a tendency to decrease in size with an increase in charge ratio from 1:1 to 10:1 (dimeric CPP) or 20:1 (monomeric CPP) (**Figure 2C**).

Figure 1. (A) Chemical structures of the monomeric and the dimeric CPP. (B) Schematic representation of NC-1 and NC-2 nanocomplexes formation. NC-1 were prepared from monomeric peptide and AMO, NC-2 from dimeric peptide and AMO. AMO: Anti-miRNA oligonucleotide; CPP: Cell-penetrating peptide; NC: Nanocomplex.

For NC-2, no NCs could be formed above a charge ratio of 10:1 ($\pm$) due to the formation of large aggregates. Incorporation of miRNA into a miR-CPP complex is intended to prevent degradation of miRNA. In spite of identical charge ratios, NC-1 and NC-2 showed significantly distinct surface charges at charge ratio 10:1 (**Figure 2D**). This gives a strong indication that at the given charge ratio both peptides form structurally different complexes. The surface charge of both NC-1 and NC-2 was only slightly affected by changes in charge ratio, ranging between 5 and 20 mV (**Figure 2D**). NC-1 showed only a slight increase in surface charge at all charge ratios (**Figure 2D**). In contrast, NC-2 showed a distinct increase in surface charge from ratio 5:1 (6 mV) to 10:1 (18.2 mV) (**Figure 2D**). To investigate the encapsulation of miRNA into NCs, a gel shift assay was performed using electrophoresis. As shown in **Figure 2E & F**, naked miRNA easily passed through the pores of a 2% agarose gel, while NCs

remained entrapped at the loading point indicating stable complex formation. In the case of NC-2 (at ratio 1:1), the complexes were not stable as free AMOs moved through the gel (**Figure 2F**).

Figure 2. Physicochemical characterization of NC-1 and NC-2 nanocomplexes. Representative size distribution of NC-1 and NC-2 complexes with charge ratios ranging from 1:1 to 20:1 are shown in (A) and (B), respectively. The mean particle size and mean ζ -potential, measured with dynamic light scattering are shown in (C) and (D). Gel shift assay for miRNA encapsulation of NC-1 (E) and NC-2 (F). Data represent mean $\pm$ standard error of the mean, $n = 3$. Statistical analysis was performed by two-tailed unpaired Student's t -test. Statistical differences are $**p < 0.01$. af: Aggregate formation; NC: Nanocomplex.

3.3.2 Increased cellular localization & transfection efficiency by NC-2 compared with NC-1

Figure 3. Uptake efficiency of NC-1 and NC-2 nanocomplexes in human pancreatic stellate cells. NCs were formed with monomeric/ dimeric CPP and FAM-labeled AMO. Flow cytometry histograms of hPSCs without transfection (control, [A]) and after exposure to NC-1 (B) and NC-2 (C) at different charge ratios. Quantitative analyses show the total uptake in hPSCs with NC-1 and NC-2 at different charge ratios (D). Histogram showing a comparison between uptake for lipofectamine-2000 complexes and NC-2 nanocomplexes at charge ratio 10:1 (E). Quantitative analysis of the total uptake of lipofectamine-2000 complexes and NC-2 nanocomplexes at charge ratio 10:1 (F). Statistical analysis was performed by two-tailed unpaired Student's *t*-test. Data represent mean $\pm$ standard error of the mean, $n=3$. Statistical differences are $**p < 0.01$. AMO: Anti-miRNA oligonucleotide; CPP: Cell-penetrating peptide; hPSC: Human pancreatic stellate cell; NC: Nanocomplex; nd: Not determined.

To investigate the uptake efficiency of the formed NCs, we performed flow cytometry for all NCs in hPSCs (**Figure 3**). NC-1 NCs at any charge ratio showed very low to no uptake in hPSCs (**Figure 3B & D**). Intriguingly, NC-2 NCs showed a remarkably increased uptake from ratios 5:1 to 10:1 (**Figure 3C & D**). Compared with NC-1, NC-2 had about 130-fold higher uptake by hPSCs at charge ratio 10:1 (**Figure 3D**). In addition, the delivery efficiency with NC-2

(10:1) was significantly higher in hPSCs compared with miRNA uptake with the gold standard transfection reagent lipofectamine-2000 (**Figure 3E & F**).

Figure 4. Uptake efficiency of NC-2 (10:1) nanocomplexes in human pancreatic stellate cells after 6 h of incubation. NC-2 were formed with dimeric CPP and FAM-labeled AMO. Histograms showing flow cytometric analyses of hPSCs (A), BJ-fibroblasts (B), Panc-1 (C), AsPc-1 (D) and MiaPaCa-2 (E) without transfection (control) and after exposure to NC-2 at the charge ratio 10:1. (F) Quantitative analyses show the total uptake in hPSCs, BJ-fibroblasts, Panc-1, AsPc-1 and MiaPaCa-2 with NC-2 at charge ratio 10:1 (colors match with histograms [A–E]). (G) Cell viability with NC-2 (10:1) and lipofectamine-2000 in hPSCs, BJ-fibroblasts, Panc-1, AsPc-1 and MiaPaCa-2 after 24 h of incubation. Statistical analysis was performed by two-tailed unpaired Student's *t*-test. Data represent mean $\pm$ standard error of the mean, *n* = 3. Statistical differences are ****p* < 0.001. AMO: Anti-miRNA oligonucleotide; CPP: Cell-penetrating peptide; hPSC: Human pancreatic stellate cell; NC: Nanocomplex.

In addition, the uptake of NC-2 (10:1) was significantly higher ($p < 0.01$) in hPSCs compared with BJ-fibroblasts and pancreatic tumor cells (i.e., Panc-1, AsPc-1 and MiaPaCa-2) (**Figure 4A–F**). Furthermore, we visualized the uptake of NC-2 in these cells using fluorescence microscopy, confirming that NC-2 NCs had much higher localization in hPSCs compared with fibroblasts (BJ-fibroblasts) and pancreatic tumor cell lines, as indicated by arrows (**Figure 5**). During their incubation, we found no cell death as confirmed by AlamarBlue assay, while lipofectamine-2000 showed severe toxicity in all cell types except Panc-1 (Figure 4G). Particularly, hPSCs and fibroblasts had the maximal toxicity ($\sim 75\%$ reduction in viability) with lipofectamine-2000.

Figure 5. *Fluorescent microscopic images of the cellular uptake of NC-2 nanocomplexes formed with FAM-labeled anti-miRNA oligonucleotide at charge ratio 10:1. Pictures were taken after 6-h incubation with hPSCs, BJ- fibroblasts, Panc-1, AsPc-1 and MiaPaCa-2. Nuclei are stained with DAPI (blue), filamentous actin with phalloidin (red). AMOs were labeled with FAM (green). Scale bar: 100 μ m. AMO: Anti-miRNA oligonucleotide; CPP: Cell-penetrating peptide; hPSC: Human pancreatic stellate cell; NC: Nanocomplex.*

3.3.3 NC-2 are partly taken up by clathrin-mediated endocytosis

Figure 6. Role of Heparan sulfate proteoglycans in uptake of NC-2 Nanocomplexes. Presence of HSPGs on the surface of hPSCs (A) and Panc-1 (B). Scale bar: 100 μ m. Histogram of hPSCs (C) and Panc-1 (D) cells after 6 h incubation with NC-2 (10:1) Nanocomplexes in the absence and presence of heparin preincubated with the cells 30 min before addition of NC-2. Quantitative analyses for the effect of heparin on the NC-2 transfection efficiency in hPSCs (E) and Panc-1 (F), respectively. Endocytosis mechanism for the uptake of NC-2 by hPSCs (G). Uptake of Nanocomplexes by hPSCs after 6 h incubation with NC-2 (10:1) Nanocomplexes incubated with nystatin, PAO and EIPA for 10 mins prior to the incubation with NC-2 (G). Statistical analysis was performed by two-tailed unpaired student t-test. Data represent mean $\pm$ SEM., n=3. Statistical differences are * p <0.05, ** p <0.01 and *** p <0.001. EIPA: 5-(N-ethyl-N-

isopropyl)-amiloride; hPSC: Human pancreatic stellate cell; HSPG: Heparan sulfate proteoglycan; NC: Nanocomplex.

Evidence exists showing that lipo-and polyplexes initiate endocytosis via the attachment to HS proteoglycans (HSPGs)^{32,33}. Fluorescent staining for heparan sulfate (HS) on the surface of hPSCs and Panc-1 cancer cells showed an increased expression of HSs on the surface of hPSCs compared with Panc-1 (**Figure 6A & B**). To prove an HSPG-mediated uptake mechanism, we performed a competition assay in which heparan was added to the culture medium to compete with HSPG for the binding of NCs. The competition assay resulted in the complete inhibition of NC-2 uptake by hPSCs and Panc-1 cells (**Figure 6C-F**). These data indicate that HSPGs are, at least partially, involved in the internalization of the NC-2 NCs by hPSCs and Panc-1. To study the mechanism of endocytosis by which the NC-2 NCs are taken up into hPSCs, we incubated hPSCs with known inhibitors for clathrin-mediated endocytosis, phenylarsine oxide³⁴, macropinocytosis, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA)³⁵, and for caveolin-dependent endocytosis, nystatin³⁶. We found that nystatin did not affect the uptake of NC-2 by hPSCs showing that caveolin-dependent endocytosis does not play a role in their uptake (**Figure 6G**). On the other hand, treatment of hPSCs with PAO and EIPA did significantly block the uptake of NC-2 by hPSCs (**Figure 6G**).

3.3.4 NC-2 mediated anti-miRNA-199a delivery inhibits hPSC activation into CAFs

We synthesized anti-miR-199a oligonucleotides with 2'-O-methyl modification with phosphodiester backbone and then prepared NCs with dimeric CPPs. Using NC-2/anti-miR199a, we were able to significantly induce mTOR in hPSCs (**Supplementary Figure 1**), and one of the target genes of miR-199a, which was previously introduced using ingenuity pathway analysis (IPA)³⁷. To differentiate hPSCs into their activated state, we incubated them with human recombinant TGF- β 1, resulting in the upregulation of the hPSC activation transcription markers Acta2, Colla1 and PDGFR- β (**Figure 7**). Interestingly, incubation of NC-2/anti-miR199a with hPSCs resulted in a significant inhibition of Acta2, Colla1 and PDGFR- β gene expression compared with the nontransfected control and scrambled AMO control (**Figure 7A-C**). Further- more, we examined the

effect on the protein expression of α -SMA and Col1 and found a clear downregulation of these markers with NC-2/anti-miR199a (**Figure 7D**).

Figure 7. Nanocomplexes of anti-miR-199a oligonucleotide and their inhibitory effects on human pancreatic stellate cells. Gene expression of *Acta-2* (A), *Col1a1* (B) and *PDGFR-β*(C) relative to house-keeping gene *RPS18* in hPSCs, activated with TGFβ, after transfection with anti-miR-199a. (D) Protein expression of α -SMA and Col1 after transfection with anti-miR-199a containing nanocomplexes. Statistical analysis was performed by two-tailed unpaired Student's *t*-test. Data represent mean $\pm$ standard error of the mean, *n* = 3. Statistical differences are **p* < 0.05; ***p* < 0.01. α -SMA: Alpha-smooth muscle actin; *Col1a1*: *Collagen1a1*; hPSC: Human-derived pancreatic stellate cell; NC: Nanocomplex.

3.3.5 hPSC treatment with NC-2/anti-miR199a inhibits the formation of 3D heterospheroids

Furthermore, to study the impact of anti-miR199a on hPSC-mediated tumor formation, we established a stroma-rich 3D spheroid assay. Using the hanging drop method, we generated heterospheroids consisting of Panc-1 and hPSCs (Figure 8A). We found that heterospheroids composed of hPSCs and Panc-1 expressed high amounts of collagen-1 when compared with Panc-1 spheroids (**Figure 8A**). To get an idea about the paracrine effects which Panc-1 exert on

hPSCs within the heterospheroids, we performed a Panc-1 conditioned medium experiment. We showed that conditioned medium collected from Panc-1 could induce hPSC differentiation as shown by the significant increase of the hPSC activation markers Acta-2 (**Figure 8B**), Colla1 (**Figure 8C**) and PDGFR- β (**Figure 8D**).

Figure 8. Effect of anti-mir-199a in tumor spheroids. (A) Images made from 6 μ m thick heterospheroid sections stained for Collagen1. Nuclei are stained with Dapi, Collagen1 was stained with Alexa 594. Scale bar = 250 μ m. Gene expression of α SMA (B), Colla1 (C) and PDGFR β (D) relative to house-keeping gene RPS18 in hPSCs, activated with Panc-1 conditioned medium. (E) Presence Calcein red labeled hPSCs in 6 μ m Panc-1/hPSC heterospheroid sections. Spheroids were transfected with Nanocomplexes containing anti-miR-199a or anti-miR-NC. Scale bar = 250 μ m. Nuclei are stained with Dapi, hPSCs were stained with calcein red-orange AM. (F) Nanocomplexes containing anti-miR-199a oligonucleotide and their inhibitory effect on the formation of heterospheroids composed of Panc-1 and hPSCs. (C) Representative pictures of heterospheroids (n=17) formed with Panc-1 and hPSCs transfected with scrambled miR and anti-miR-199a. (F) Dotted line represents the contour of the spheroid formed with miR-NC treated hPSCs. Scale bar = 250 μ m. (G) Effect of conditioned medium of anti-miR-199a treated hPSCs on Panc-1 cell growth over 48 hours. Statistical analysis was performed by two-tailed unpaired student t-test. Data represent mean $\pm$ SEM., n=3. Statistical differences are *** $p < 0.005$.

To evaluate if NCs carrying anti-miR199a could inhibit the growth of 3D heterospheroids composed of hPSCs and Panc-1 tumor cells, we transfected hPSCs with NC-2/anti-miR199a and used them for spheroid formation. After 7 days, we found that heterospheroids composed of Panc-1 and hPSCs transfected

with anti-miR-199a formed smaller spheroids compared with the heterospheroids containing hPSCs transfected with scrambled miRNA (**Figure 8F**). To prove that the smaller spheroids do not consist of cancer cells only, we have labeled hPSCs transfected with NC-2/anti-miR199a with Calcein red AM prior to spheroids formation and still found these cells within the spheroids after 7 days (**Figure 8E**). Interestingly, there were similar number of hPSCs in anti-miR-199a transfected spheroids compared with the control spheroids. In contrast, the number of tumor cells was much lesser in the spheroids formed with anti-miR199a-hPSCs compared with the control spheroids (**Figure 8E**). To get an indication about the paracrine effects which hPSCs exert on Panc-1 cell growth, we performed a conditioned medium experiment (**Figure 8G**). We showed that conditioned medium collected from the activated hPSCs stimulated the growth of Panc-1 tumor cells compared with conditioned medium from normal hPSCs (**Figure 8G**). When hPSCs were transfected with anti-miR199a, the cell growth of Panc-1 tumor cells was inhibited (**Figure 8G**).

3.4 Discussion

In this study, we have developed a novel strategy to deliver AMO to hPSCs, the key precursor cells of CAFs in pancreatic cancer. A dimeric form of an arginine-rich CPP was synthesized and used for preparing NCs with AMOs. Intriguingly, these NCs showed very high uptake by hPSCs compared with the NCs with the monomeric forms. Although branching or multivariant approaches may enhance CPP-mediated uptake, our dimeric CPP-based NCs were highly specific to hPSCs compared with normal fibroblasts or pancreatic tumor cells. To this end, we examined different mechanisms for uptake by hPSCs. Then, we applied these NCs for specific delivery of an AMO against miR-199a (induced in activated hPSCs and CAFs³⁷) to hPSCs in 2D monocultures and 3D co-cultures. Interestingly, delivery of anti-miR-199a into hPSCs inhibited their differentiation into myofibroblasts and hPSC-mediated tumor cell growth.

Evidence suggests that miRNAs are key endogenous regulators of differentiation of CAF and their functions in the tumor microenvironment⁴. Reversal of upregulated or downregulated miRNA using anti-miRNA or miRNA-mimics, respectively, seems to be a highly interesting approach to inhibit CAF-mediated tumor- supporting effects⁴. However, a major challenge is the specific delivery of these single stranded anti-miRNA or miRNA-mimic oligonucleotides into the target cells⁵. In this study, we designed a dimeric form of an arginine- rich CPP using a disulfide bridge to attain a longer and more flexible chain which allows

for a better interaction with AMO. In addition, these complexes at charge ratio 10:1 have a higher availability of CPP at the surface of the NCs to achieve better interactions with the cellular surface for their efficient uptake. Furthermore, NC-2 possessed a higher surface charge compared with NC-1 at charge ratio 10:1 which suggests that more CPPs are available on the surface of NC-2.

The cellular uptake study in hPSCs showed that dimeric CPP-based NC-2 were 130-fold higher taken up by hPSCs compared with monomeric CPP-based NC-1 (**Figure 3**). These data clearly showed the benefit of our dimeric form of CPP over the monomeric form. Although studies have shown that branching or multimeric forms of CPP may enhance their cellular uptake^{38,39}, in the present study we surprisingly found that NC-2 complexes were not only enhancing general cellular uptake but also very specifically enhanced cellular uptake in hPSCs compared with normal fibroblasts and different pancreatic tumor cells (Panc-1, MiaPaCa-2 and AsPc-1) (**Figures 4 & 5**). This preferential uptake by hPSCs led us to explore this observation further. Fluorescent staining revealed increased expression of HSs on the surface of hPSCs compared with Panc-1 cancer cells (**Figure 6A & B**). Competition studies with heparan indicate that NC-2 are taken up by an HSPG-dependent mechanism (**Figure 6C-F**). In the case of arginine-rich CPPs, it has been proposed that the interaction of peptides with membrane-associated proteoglycans triggers the induction of actin organization and macropinocytosis⁴⁰. The affinity for the interaction with HSPG seems to be dependent on the structure of arginine-rich CPPs⁴⁰. Additionally, it has been found that arginine-rich CPPs bind to HSPGs with binding constants of nanomolar concentration and therefore can be considered as receptors for intracellular uptake^{32,41-43}. Blocking of NC-2 uptake with PAO and EIPA did significantly block the uptake of NC-2, indicating that next to macropinocytosis, clathrin-mediated endocytosis plays a role in the uptake of NCs by hPSCs (**Figure 6G**). The receptor (clathrin) mediated uptake of NCs can, at least up to some extent, explain the preferential cellular uptake of NC-2 NCs by hPSCs compared with BJ-fibroblasts, Panc-1, MiaPaCa-2 and AsPc-1 cells. Likely, this is due to the NCs interacting with HSPGs attached to trans- membrane receptors on the cellular surface of hPSCs.

Recently, miRNA-199a was identified as a novel therapeutic target in pancreatic tumor stromal fibroblasts in patients³⁷. It was shown, that miRNA-199a was induced in patient-derived CAFs and activated hPSCs and regulated the differentiation of these cells as well as hPSC-induced protumorigenic effects³⁷. In this study, we showed that NC-2 containing active AMOs against miRNA-

199a could inhibit hPSC differentiation at both mRNA and protein level, as shown with the CAF markers α -SMA, Col-1a1 and PDGFR- β . In addition, we demonstrated that delivery of anti-miR-199a leads to activation of the direct target gene mTOR in hPSCs (**Supplementary Figure 1**), implying that the effects observed on hPSCs are miR-199a related. These data clearly demonstrate the feasibility of our approach enabling the transfection of an active AMO into hPSCs, leading to the inhibition of hPSC activation markers.

Within the tumor microenvironment, activated hPSCs and CAFs stimulate tumor cell growth by secreting cytokines and thereby induce tumor progression. Our 3D heterospheroidal system mimics the interaction between tumor cells and hPSCs, as shown with the presence of Col1 staining as marker for activated hPSCs (**Figure 8A**). Our conditioned medium studies also confirmed that hPSCs get activated by factors secreted by Panc-1 (**Figure 8B-D**). The inhibition of the growth of 3D heterospheroids, composed of hPSC and Panc-1 tumor cells, after anti-miR-199a delivery into hPSCs clearly demonstrated that inhibition of anti-miR-199a using our novel NC-2 is capable of intervening into hPSC-induced tumor-inducing effects. A previous study has shown that transfection of anti-miR-199a may reduce the hPSCs growth³⁷. From the fluorescent images of spheroids (**Figure 8E**), it is apparent that only number of tumor cells was reduced in spheroids formed with anti-miR-199a hPSCs compared with control spheroids. To better understand whether the 3D spheroid growth inhibitory effects of the targeted anti-miR199a were due to the inhibition of hPSC-mediated Panc-1 growth but not majorly due to hPSC growth itself, we performed additional experiments using hPSC conditioned medium. We found that conditioned medium obtained from TGF β -activated hPSCs induces Panc-1 tumor cell growth, while the activated hPSCs treated with anti-miR-199a did not induce the growth of Panc-1 tumor cells, confirming that delivery of anti-miR-199a indeed inhibits hPSC-mediated tumor cell growth both in 2D monocultures and 3D co-cultures.

3.5 Conclusion

In conclusion, we have developed a novel dimeric CPP-based self-assembling delivery system to specifically deliver AMOs to hPSCs compared with tumor cells. We also demonstrated that our miRNA delivery system is functional in delivering active miRNA to hPSCs and in inhibiting their differentiation into tumor-promoting CAF phenotype. Altogether, this study presents a highly

efficient peptide-based anti-miRNA delivery system to hPSCs to inhibit hPSC-mediated protumorigenic activity.

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3.8 Supplementary data

Figure S1. Nanocomplexes of anti-miR-199a oligonucleotides induce the target gene mTOR of miR-199a in human hPSCs. Gene expression of mTOR relative to house-keeping gene RPS18 in hPSCs. Statistical analysis was performed by two-tailed unpaired student t-test. Data represent mean $\pm$ SEM, $n=3$. Statistical differences are ** $p<0.01$.

Chapter 4 - Reprogramming tumor stroma using an endogenous lipid lipoxin A4 to treat pancreatic cancer

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Abstract

Pancreatic stellate cells (PSCs) are the precursors of cancer-associated fibroblasts (CAFs), which potentiate pancreatic tumor growth and progression. In this study, we investigated whether Lipoxin A4 (LXA4), an endogenous bioactive lipid, can inhibit the differentiation of human PSCs (hPSCs) into CAF-like myofibroblasts and thereby hPSC-induced pro-tumorigenic effects. LXA4 significantly inhibited TGF- β -mediated differentiation of hPSCs by inhibiting pSmad2/3 signalling. Furthermore, treatment with LXA4 abolished the paracrine effects (proliferation and migration of Panc-1 tumor cells) of hPSCs in vitro. These data demonstrated that LXA4 can interrupt pro-tumoral paracrine signalling of hPSCs. Furthermore, LXA4 treatment significantly decreased the size and growth rate of 3D-heterospheroids comprised of hPSC and Panc-1 and these effects were exhibited due to inhibition of hPSC-induced collagen1 expression. In vivo, we examined the therapeutic efficacy of LXA4 in a co-injection (Panc-1 and hPSCs) subcutaneous tumor model. Intriguingly, LXA4 significantly abolished the tumor growth (either injected intratumor or intraperitoneally), attributed to a significant reduction in fibrosis, shown with collagen1 expression. Altogether, this study proposes LXA4 as a potent inhibitor for hPSCs which can be applied to reprogram tumor stroma in order to treat pancreatic cancer.

4.1 Introduction

Annually, around 90,000 people in Europe and 45,000 people in the U.S. get diagnosed with pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer [1]. Patients with PDAC have a 1 year survival rate of 20%, whereas the 5-year survival rate decreases to 7%¹. In most PDAC, a surgical resection is not feasible, making radiotherapy or cytotoxic chemotherapy the golden standard treatment. However, these treatments are not effective and result in a survival benefit of only a few months^{2,3}. One reason of the treatment failure is the abundant desmoplastic reaction in PDAC, also called the tumor stroma, which supports cancer progression, invasion and metastasis⁴. Novel therapeutic approaches for the treatment of PDAC aim to modulate the cellular components of the tumor stroma. Several different studies have shown an anti-tumoral effect by modulating the tumor stroma^{5,6}, whereas unexpectedly a complete depletion of the stroma genetically was shown to promote tumor growth⁷. Therefore, it has been suggested to dampen the activity of tumor stroma instead of its total depletion to gain therapeutic benefits⁸.

Human pancreatic stellate cells (hPSCs) are the main precursors of pancreatic cancer-associated fibroblasts (CAFs), the key drivers of the pancreatic tumor stroma^{5,9,10}. Quiescent hPSCs can be found within the connective tissue of the pancreas in low numbers and secrete only small amounts of extracellular matrix (ECM) proteins (e.g. collagen)¹¹. In PDAC, quiescent hPSCs become activated, lose their cytoplasmic lipid storing capacity and start secreting high amounts of ECM components, leading to fibrosis which ultimately presents as a barrier for tumor drug penetration. In addition, differentiated hPSCs secrete various cytokines and growth factors which stimulate tumor cells and other stromal cells in favour of tumor progression¹². Therefore, inhibition of hPSC activation might be beneficial for the treatment of pancreatic cancer.

Lipoxins are part of a family of inflammation resolving endogenous lipid mediators which are locally secreted by immune cells such as neutrophils and macrophages in response to infection, injury or inflammatory stimuli¹³⁻¹⁶. Lipoxins are synthesized from membrane arachidonic acid via biochemical synthesis by two major routes. The first route of synthesis starts in platelets where leukotriene A₄ is converted into lipoxin by 12-LO. The second major route involves the action of either 5-LO in neutrophils and 15-LO in erythrocytes and reticulocytes. Arachidonic acid is converted into 15-hydroperoxyeicosatetraenoic acid (15-HPETE), which is then converted into lipoxin A and lipoxin B¹⁷. Based on an aspirin-triggered reaction in which

aspirin acetylates cyclooxygenase-2 (COX-2) to form 15R-HPETE, and is eventually metabolized by 5-LO epi-lipoxin, lipoxin A4 (LXA4) and lipoxin B4 (LXB4) are formed^{18,19}. Lipoxins and epi-lipoxins function by binding to the high-affinity G protein-coupled lipoxin A4 (LXA4) receptor formyl peptide receptor 2 (FPR2)/ ALX¹⁴. Via this route, LXA4 can resolve inflammation already at low nanomolar concentrations and additionally activates anti-bacterial mechanisms^{20,21}.

There are little evidences that LXA4 may possess anti-fibrotic effects. In mesangial cells in kidney, it inhibited platelet-derived growth factor (PDGF)–dependent TGF- β production, the expression of fibrosis promoting genes in renal mesangial cells²² and Akt/PKB activation and cell cycle progression in mesangial cells²³. Additionally, LXA4 attenuated experimental renal fibrosis²⁴ and inhibited epithelial to mesenchymal transition of renal epithelial cells in proximal tubules²⁵. Also, LXA4 has been shown to inhibit connective tissue growth factor-induced proliferation and TGF- β -dependent pro-fibrotic activity in human lung (myo)fibroblast^{13,26}. Yet, there is no study showing the inhibitory effects of LXA4 on hPSC differentiation and its impact on pancreatic tumor growth.

Considering the anti-fibrotic activity of LXA4, in this study, we hypothesized that LXA4 might be an interesting biomolecule to inhibit PSCs activation and their pro-tumorigenic effects. We show that the LXA4 specific receptor FPR2/ALX is overexpressed in activated hPSCs. Furthermore, we demonstrate the inhibitory effect of LXA4 on the PSC activation, migration and then PSC-induced paracrine effect on pancreatic cancer cells. Next, we showed the inhibitory effects of LXA4 on the growth and extracellular matrix (ECM) deposition in stromal-rich 3D-heterospheroids. Eventually, we examined the therapeutic efficacy of LXA4 *in vivo* in a co-injection tumor model which resulted in a reduced tumor growth and intra-tumoral ECM deposition after the treatment with LXA4.

4.2 Materials and methods:

4.2.1 Cells

Primary human pancreatic stellate cells (ScienCell, Carlsbad, USA), were cultured in complete Stellate Cell Medium (supplemented with 2 % FBS, 1 % Penicillin/Streptomycin and 1 % Stellate Cell Growth Supplements (SteCGS)) (ScienCell). Panc-1 cancer cells were cultured in Dulbecco's Modified Eagles

medium (DMEM) High Glucose (4.5 g/l) with L-Glutamine (GE Healthcare, Vienna, Austria) supplemented with 10 % FBS (Lonza, Verviers, Belgium), 100 µg/ml penicillin/streptomycin (Sigma Aldrich). AsPc-1 cells were cultured in complete RPMI Medium (GE Healthcare, Vienna, Austria) supplemented with 10 % FBS (Lonza), 100 µg/ml penicillin/streptomycin (Sigma). The cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere

4.2.2 Western blot

To evaluate the expression of the LXA4-specific surface receptor FPR2 and the hPSC activation marker α -SMA, hPSCs were seeded into a 12 well plate at a seeding density of 40.000 cells/well. The next day hPSCs were starved for 24 h and subsequently activated with 5 ng/ml TGF- β for 48 h. To evaluate the effect of LXA4 on TGF- β -mediated Smad 2/3 and phosphorylated Smad 2/3 (pSmad2/3) levels in hPSCs, cells were seeded into a 12 well plate at a seeding density of 40.000 cells/well. The cells were starved for 24 h, treated with 10 nM LXA4 (Biomol, Hamburg, Germany) and activated with 5 ng/µl TGF- β for 30 min. In general, cells were lysed using 1× SDS-lysis buffer, cell lysis was centrifuged at 10,000 g for 10 min, and the supernatant was collected for Western blot analysis. To analyse the expression of MMP-2 in conditioned medium, 1 ml conditioned medium was freeze dried using an Il-Shin TFD5503 Freeze Dryer (Scala Scientific, Ede, The Netherlands) and afterwards resuspended in 100 µl TBS (Thermo Scientific, Rockford, USA). Conditioned medium was mixed with Sample Reducing agent (Life Technologies, Carlsbad, USA) and LDS Sample Buffer (Life Technologies) and incubated at 95 °C for 10 min. Protein lysates and conditioned medium was loaded on a 10% Tris-Glycine gel (Thermo Scientific) and transferred onto PVDF membranes (Thermo Scientific). The blots were incubated with the required primary antibody (Supplementary Table 1) and incubated overnight at 4 °C, followed by incubation with species specific horseradish peroxidase (HRP) conjugated secondary and tertiary antibody for 1 h at RT. Proteins were detected with Pierce™ ECL Plus Western Blotting Substrate kit (Thermo Scientific) and exposed to FluorChem™ M System (ProteinSimple, San José, USA). The protein levels were quantified using Image J software (NIH, MD). To evaluate the amount of Smad 2/3 phosphorylation a pSmad 2/3/Smad 2/3 ratio was calculated.

4.2.3 Immunocytochemistry

To evaluate the effect of LXA4 on the expression of α SMA and collagen1 (Col1), hPSCs were seeded into a 24 well plate at a seeding density of 5.000 cells/well. The cells were starved, treated with 0.1, 1 and 10 nM LXA4 and

TGF- β activated for 48 h. Next, cells were fixed and immunostained for α SMA, and collagen1 as described elsewhere [27]. Stained images were quantified for their positively stained area using ImageJ software.

To evaluate the effect of LXA4 on Smad2/3 and pSmad2/3 expression, hPSCs were seeded into a 24 well plate at a seeding density of 10.000 cells/well. The cells were starved and activated with TGF- β as described previously. After 30 min of incubation with TGF- β , cells were washed with phosphate buffered saline (PBS) (Lonza, Verviers, Belgium) and fixed with 4% paraformaldehyde (Sigma Aldrich) at room temperature for 10 min. Next, cells were washed 3 times with PBS and permeabilized with ice cold HPLC grade methanol (Fisher Scientific) at -20°C for 10 min. After washing 3 times with PBS cells were blocked with blocking buffer consisting of 1 vol % bovine-serum albumin (BSA) (VWR, Radnor, PA, USA) + 0.3 vol % Triton-X (Sigma Aldrich) in PBS for 1 h. Subsequently, the cells were incubated with monoclonal anti-Smad2/3 or monoclonal anti-pSmad2/3 primary antibodies diluted in blocking buffer and incubated over night at 4°C . Next, cells were washed 3 times with PBS and incubated with fluorescent secondary antibodies diluted in blocking buffer for 1 h at RT. Cells were washed once with PBS and stained for DAPI by incubation with NucBlue Fixed Cell Stain ReadyProbes Reagent (Life Technologies), diluted in PBS as described in the manufacturer's instructions, for 5 min. The NuCBlue solution was replaced with PBS and the cells imaged for Alexa Fluor 488 (ex. 495/em. 520) and DAPI (ex. 360/em. 460) using an EVOS FI fluorescent microscope (Life Technologies). Staining of pSmad2/3 was quantified by counting the total number and the number of pSmad 2/3 positive nuclei.

4.2.4 F-Actin staining

To analyse the effect of LXA4 on the F-actin organization of TGF- β activated hPSCs, hPSCs were seeded into 24 well plates at a seeding density of 2.500 cells/well. After 24 h of incubation with 10 nM LXA4 and / or 5 ng/ml TGF- β , cells were washed three times with PBS and subsequently fixed in PBS containing 10% formaldehyde (Sigma Aldrich) for 15 min. After permeabilizing the cells with 0.1 M Triton X-100 (Sigma Aldrich) for 5 min, F-actin was stained with phalloidin (Life Technologies) at a concentration of 250 ng/ml for 30 min. Next, cells were washed with PBS and imaged for phalloidin at (ex. 540/em. 565) using an EVOS FI fluorescent microscope (Life Technologies).

4.2.5 Quantitative real time PCR

To evaluate the effect of LXA4 on TGF- β -mediated hPSCs activation, cells were seeded into a 12 well plate at a seeding density of 40.000 cells/well. The cells were starved for 24 h, treated with 10 nM LXA4 and activated with 5 ng/ μ l TGF- β . 24 h after activation total RNA was isolated using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich) and the RNA amount was measured by a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). Subsequently, cDNA was synthesized with iScript™ cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). 10 ng cDNA were used for each PCR reaction. The real-time PCR primers (Table 1) were purchased from (Sigma Aldrich). Quantitative real time PCR was performed with the 2x SensiMix SYBR and Fluorescein Kit (Bioline GmbH, Luckenwalde, Germany) using a BioRad CFX384 Real-Time PCR detection system (BioRad). Gene expression levels were normalized to the expression of the house-keeping gene RPS18.

Table 1. Sequences of forward and reverse primers used during real-time PCR.

Gene	Forward Primer	Reverse Primer
RPS18	TGAGGTGGAACGTGTGATCA	CCTCTATGGGCCCCGAATCTT
Acta-2	CCCCATCTATGAGGGCTATG	CAGTGGCCATCTCATTTTCA
Collagen1 α 1	GTA CTGGATTGACCCCAACC	CGCCATACTCGAACTGGAAT
Collagen3 α 1	AAGAAGGCCCTGAAGCTGAT	GTGTTTCGTGCAACCATCCT
PDGF β R	AGGCAAGCTGGTCAAGATCT	GCTGTTGAAGATGCTCTCCG
MMP2	AGGAGGAGAAGGCTGTGTTC	CTCCAGTTAAAGGCGGCATC
WNT2 β	TTTAGGTGCTGGTGGGAGAG	CTCTTCTTCACCTGGGAGCA
ID1	TTGGGCTGGATAAAACCCCT	GCAACATCCGGCATAACTGT
NSDHL	ATGCTATGGAGAGGACCGTG	CAGAAGGAGTCACAGGCTCA
INSIG1	GGCAGCTTCCCAAGTATTCG	CTACCTCCTTTGGGCACTGA
CXCL12	TGCCCTTCAGATTGTAGCCC	GCGTCTGACCCTCTCACATC
CTGF	GTTTGGCCCCAGACCCAACTA	GGCTCTGCTTCTCTAGCCTG

4.2.6 Conditioned media

To collect hPSC conditioned medium, cells were seeded into a 12 well plate at a seeding density of 40.000 cells/well. As previously described, cells were starved, treated with LXA4 and activated with TGF- β for 24 h. Next, the cells were washed 3 times with serum-free medium. After 48 h of incubation with serum-free medium the medium was collected for use in conditioned medium experiments.

4.2.7 Cell proliferation assay

hPSCs were seeded into a 96 well plate at a density of 2.500 cells/well. The next day, hPSCs were treated with 10 nM of LXA4. Cell number was monitored every 24 h over a time period of 72 h. To monitor the cell number, 10 μ l of Alamar Blue dye (Invitrogen, Carlsbad, USA) in 100 μ l of hPSC growth media was added per well. After 4 h the fluorescent signal was measured using a VIKTOR™ plate reader (Perkin Elmer, Waltham, Massachusetts). To evaluate the effect of hPSC conditioned media on Panc-1 and AsPc-1 cell growth, Panc-1 and AsPc-1 were seeded in a 96 well plate at a density of 5000 cells/well. 24 h after seeding the previously collected condition media was applied to the cells. Cell number was monitored at 0 h, 24 h and 48 h as described previously.

4.2.8 Wound healing assay

To evaluate the effect of LXA4 on hPSC migration, cells were seeded into a 24 well plate at a seeding density of 50.000 cells/well. As previously described cells were starved, treated with LXA4 and activated with TGF- β for 24 h. A scratch was made on the culture plate using a 200 μ l pipette tip fixed in a custom-made holder. Next, cells were washed and incubated with fresh serum-free media. Images were captured at $t = 0$ h and $t = 12$ h, with an EVOS microscope. Images were analysed by Image J software to calculate the area of the scratch and represented as percentage of wound closure.

4.2.9 Transwell migration Assay

To evaluate the effect of hPSC conditioned media on Panc-1 and AsPc-1 migration, Panc-1 and AsPc-1 were seeded into the upper chamber of a 24 well Transwell insert (Sigma Aldrich) at a seeding density of 50.000 cells/well. After 24 h, the cells were fixed with ice cold methanol (Fisher Scientific) for 10 min. Next, the cells were incubated with 0.1% Crystal Violet solution (Sigma Aldrich) in 25% methanol (Fisher Scientific) for 10 min. After washing the cells with MilliQ water, cells from the upper compartment were removed with a cotton swap and the inserts were allowed to dry. The migrated cells were imaged with a Nikon microscope (Nikon Eclipse E400, Tokyo, Japan). Images were analysed by Image J software to calculate the positively stained area.

4.2.10 Spheroid formation assay

3D-heterospheroids containing a mixture of hPSCs and Panc-1 cells were prepared using the hanging drop method. hPSCs and Panc-1 were trypsinized and suspended in their respective culture medium to a concentration of 300.000 cells/ml. The hPSCs and Panc-1 cell suspensions were mixed 1:1 (v/v).

For spheroid formation, a drop of 20 μ l containing 6000 cells were dispensed onto a lid of a round bottom suspension culture 96-well plate (Greiner BioOne, Alphen aan den Rijn, The Netherlands). Next, the lid was inverted and placed onto the 96-well plate. Wells were filled with 150 μ l PBS for humidity. For spheroids prepared with LXA4-treated hPSCs, hPSCs were seeded, starved for 24 h and treated with 10 nM LXA4 for an additional 24h. The spheroids were grown for eight days. Spheroids which were LXA4-treated after formation were allowed to form for 4 days. Next, 10 μ l of 20 nM LXA4 was directly added into the droplet. The spheroids were grown for an additional 8 days. Spheroids were imaged under an inverted microscope, and their area was measured digitally using ImageJ software.

4.2.11 Immunohistochemical staining of 3D-heterospheroids

For sectioning 3D-heterospheroids were collected, embedded in Cryomatrix (Thermo Scientific) and frozen at - 80 °C. Afterwards, spheroids were cut into 6 μ m thick sections using a Cryotome FSE (Thermo Scientific). The spheroids collected on microscope slides were rehydrated in PBS for 5 min before fixation in 4% paraformaldehyde (Sigma Aldrich) for 30 min. Next, the slides were washed 3 times with PBS (5 min each). The edges of the slides were marked with a hydrophobic Pap-pen (Life Technologies). After washing, the cells were permeabilized with 0.1 vol % Triton-X (Sigma Aldrich) in PBS for 15 min and washed again. Slides were incubated with the primary antibody (Supplementary Table 2) diluted in PBS overnight at 4 °C. The next day, the slides were washed 3 times with PBS before the secondary fluorescent antibody (see Supplementary Table 2) diluted in PBS supplemented with 5 vol% of normal human serum (Thermofisher Scientific) were added. Finally, the slides were washed and mounted with fluoroshieldTM with DAPI (Sigma Aldrich) for staining of nuclei. Sections have been imaged using a Hamamatsu NanoZoomer Digital slide scanner 2.0HT (Hamamatsu Photonics, Bridgewater NJ).

4.2.12 Animal study

Six-week-old male CB17 SCID mice (Janvier Labs, Le Genest-Saint-Isle, France) were subcutaneously injected with a mixture of 2×10^6 Panc-1 and 4×10^6 of hPSCs. Tumor formations and volumes were assessed every 3–4 days. Tumor volumes were calculated using a standard formula [volume = $1/2$ (length x width²)]. The treatment was started 9 days after injection of the tumors. Six tumor-bearing mice per treatment groups were taken and injected with either vehicle or LXA4 twice a week. Of each group half were injected intraperitoneal

(40 µg/kg) and another half local intratumoral (4 µg/kg). At the end of the experiments, animals were sacrificed under anesthesia, tumors were harvested and immediately snap frozen in cold 2-methyl butane (Fisher Scientific). Frozen tumors were stored at -80 °C until analysis.

4.2.13 Immunohistochemical staining of mouse tumors

Mouse tumors were embedded in Cryomatrix (Thermo Scientific) and frozen at - 80 °C. Afterwards, spheroids were cut into 6 µm thick sections using a Cryotome FSE (Thermo Scientific). The tumor section were dried and fixed in acetone (Fisher Scientific) for 10 min. The edges of the slides were marked with a hydrophobic Pap-pen (Life Technologies). Next, the tumor sections were rehydrated in PBS for 10 min. Tumor sections were incubated with the primary antibody (Supplementary Table 2) diluted in PBS overnight at 4 °C. The next day, the slides were washed 3 times with PBS before the secondary fluorescent antibody or HRP-labeled antibody (Supplementary Table 2) diluted in PBS supplemented with 5 vol% of normal mouse serum (Thermofisher Scientific) were added (normal human serum was used for α-SMA staining). Staining with HRP-labeled secondary antibodies was continued as described elsewhere ²⁷. For staining with fluorescent antibodies, the slides were washed and mounted with fluoroshield™ with DAPI (Sigma Aldrich) for staining of nuclei. Sections have been imaged using a Hamamatsu NanoZoomer Digital slide scanner 2.0HT (Hamamatsu Photonics, Bridgewater NJ).

4.2.14 Immunohistochemical staining of human tumors

Human paraffin embedded PDAC sections were deparaffinized in xylene, rehydrated in serially diluted alcohol solutions, followed by demineralized water. Antigen retrieval was performed by at 80 °C using Tris buffer (pH 9.0, Dako, Glostrup, Denmark) for 16 h. To block endogenous peroxidase, slides were incubated in 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 20 min. Next, slides were incubated for 2 h with an antibody against α-SMA (A2547 Sigma, the Netherlands), followed by incubation with the secondary antibody for 60 min at room temperature. The immunohistochemical staining was visualised using 3,3-diaminobenzidine tetrahydrochloride solution (Dako) for 5–10 min resulting in a brown staining and then counterstained with hematoxylin, dehydrated and finally mounted in vectamount.

4.2.15 Graphs & statistical analysis

All graphs were made using GraphPad Prism Vol.5 (GraphPad Software Inc., San Diego, CA). All values are expressed as a mean ± standard error of the mean

(SEM). Statistical significance of the results was performed by either a two-tailed unpaired student's t-test for comparison of two treatment groups or a one-way ANOVA to compare multiple treatment groups. Differences were considered significant for a p-value of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, respectively.

4.3. Results

4.3.1 LXA4 expression is induced in TGF- β activated hPSCs

Fig. 1. Structure and protein expression of the Lipoxin A4 (LXA4) specific surface receptor FPR2 on hPSCs. Structure of the LXA4-specific surface receptor FPR2 displaying the binding site of LXA4 (A). Protein expression of the LXA4-specific surface receptor FPR2 (B, C) and the activation marker α -SMA (B, D) in hPSCs and hPSCs activated with TGF- β for 48 h (B). Data represents mean $\pm$ SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. Statistical differences are * $p < 0.05$, ** $p < 0.01$.

LXA4 binds to the extracellular domain of the N-formyl-peptide receptor 2 (FPR2) receptor as shown in Fig. 1 A. To achieve the effect of LXA4 on hPSCs, it is crucial to confirm whether these cells express the FPR2 receptor in their quiescent and active state. Therefore, we activated hPSCs with TGF- β for 48 h and examined the protein expression of FPR2 using western blot analysis. As shown in Fig. 1 B, the expression of FPR2 receptor was induced by about 6.5-fold in the TGF- β -activated hPSCs compared to the non-activated form (Fig. 1 B, C). To confirm the activation of hPSCs with TGF- β we have performed a western blot for α -SMA, showing the significant induction of this CAF marker upon TGF- β treatment (Fig. 1 B, D).

4.3.2 LXA4 inhibits TGF- β induced PSC differentiation

As shown in Fig. 2A and D, after activation with TGF- β , hPSCs attained a myofibroblast phenotype, as can be seen with their high expression of α -SMA and collagen1 (Col1) and stretched morphology.

Fig. 2. Lipoxin A4 effect on TGF- β mediated hPSC differentiation. Immunostaining showing the inhibitory effect of LXA4 (0.1, 1, 10 nM) on the protein expression of α -SMA and Col1 (A). Scale bar = 200 μ m. Quantification of α -SMA (B) and Col1 (C) staining for the respective concentrations of LXA4. F-actin staining showing morphological changes in TGF- β activated and LXA4-treated and TGF- β activated compared to normal hPSCs (D). Control contains 0.001% ethanol. Data represent mean $\pm$ SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * p < 0.05, ** p < 0.01, *** p < 0.001.

Treatment with LX4 not only significantly inhibited the protein expression of α -SMA and Col1 in a concentration dependent manner, but also inhibited the change in the morphology (Fig. 2 A, D). The quantitation of stainings confirmed the reduction in the expression of both α -SMA and Col1 with the most profound effects at a concentration of 10 nM (Fig. 2 B, C).

Fig. 3. Lipoxin A4 effect on TGF- β mediated hPSC differentiation, viability and migration potential. Gene expression of hPSC activation markers encoding growth factors (CTGF), membrane receptors (PDGF β R), chemokines (IL-6 and CXCL12), enzymes (MMP2), proteins involved in cholesterol production and homeostasis (IDI1, INSIG1 and NSDHL), intracellular proteins (α -SMA, Wnt2 β) and ECM proteins (Col1 α 1, Col3 α 1) displaying the inhibitory effect of 10 nM LX4 after treatment with 5 ng/ml TGF- β LX4 (A). Mitochondrial activity of hPSCs treated with 10 nM Lipoxin A4 and EtOH control after 24, 48 and 72 h of incubation (B). Representative microscopic images and quantification showing the effect of LX4 (10 nM) on the migration of hPSCs and hPSCs activated with TGF- β (C). Scale bar = 500 μ m. Control contains 0.001% ethanol. Data represent mean $\pm$ SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * p < 0.05, ** p < 0.01, *** p < 0.001.

Interestingly, LXA4 at 10 nM concentration significantly inhibited the TGF- β -induced expression of genes encoding for the CAF phenotype, including growth factors (CTGF and CXCL12), membrane receptors (PDGF β R), cytokines inducing cancer cell growth (IL-6), enzymes facilitating increased ECM turnover and altered ECM deposition (MMP2), genes involved in cholesterol production and homeostasis (IDI1, INSIG1, NSDHL), intracellular proteins (Acta-2 (encoding for α -SMA), Wnt2B) and ECM proteins (Col1 α 1, Col3 α 1) (Fig. 3 A). Additionally, we detected a significant reduction in TGF- β -induced expression of secreted MMP-2 in the conditioned medium of hPSCs treated with 10 nM of LXA4 (Supplementary Fig. 2). Of note, treatment of hPSCs with 10 nM LXA4 over a period of 72 h exerted no toxic effects on their metabolic activity (Fig. 3 B). Next, we examined the effect of LXA4 on the migration of hPSCs using a scratch (wound healing) assay. The migration potential in TGF- β -activated hPSCs was not induced compared to untreated hPSCs (Fig. 3 C). LXA4 led to a significant inhibition of the wound closure in both non-activated and TGF- β -activated hPSCs compared to control cells (Fig. 3 C). These data indicate that LXA4 inhibits the activation of hPSCs with TGF- β to attain the CAF-like myofibroblast phenotype.

4.3.4 LXA4 inhibits TGF- β induced smad 2/3 nuclear translocation and phosphorylation

Since LXA4 inhibited the TGF- β -mediated activation of hPSCs, we examined the effect of LXA4 on the TGF- β signaling pathway. After binding to its receptor, TGF- β activates the Smad 2/3 pathway, whereby Smad 2/3 gets phosphorylated and locates within the nucleus. As shown in Fig. 4 A using immunofluorescent microscopy, in non-activated hPSCs, Smad2/3 was localized in the cytoplasm but not in the nuclei, while after 30 min incubation with TGF- β , Smad 2/3 translocated into the nuclei (Fig. 4 A). Treatment with LXA4 reduced the translocation into the nuclei (Fig. 4 A). To confirm the activation state of Smad2/3 and quantify this, we examined the phosphorylated Smad2/3 (pSmad2/3) using immunofluorescent staining which clearly showed a significant translocation of pSmad2/3 into the nuclei of hPSCs upon TGF- β activation compared to untreated cells (Fig. 4 A). Importantly, the nuclear translocation of pSmad 2/3 was significantly inhibited by LXA4, as shown in the immunofluorescent pictures and quantitative data (Fig. 4 A, B). Additionally, we have performed a Western Blot to analyse the protein expression of Smad 2/3 and pSmad 2/3 (Supplementary Figure 1 A) and found a significant reduction in the pSmad 2/3/Smad 2/3 ratio (Supplementary Figure 1 B), confirming the

inhibition of Smad 2/3 phosphorylation, which we previously had observed in immunofluorescent staining.

Fig. 4. Effect of LXA4 on Smad 2/3 phosphorylation in TGF- β activated hPSCs. Immunofluorescent staining of Smad 2/3 and pSmad 2/3 in hPSCs, TGF- β activated hPSCs treated with control or 10 nM LXA4 (A). Scale bar = 50 μ m. Quantification of fluorescent pSmad 2/3 staining showing the percentage of pSmad 2/3 positive nuclei (B). Control contains 0.001% ethanol. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. ** $p < 0.01$.

4.3.5 LXA4 inhibits hPSC-mediated paracrine effects on tumor cells

After confirming the direct effect of LXA4 on hPSCs, we investigated the hPSC-induced paracrine effect on the growth and migration potential of human pancreatic tumor cells (Panc-1 & AsPc-1). To study that, we collected conditioned media from hPSCs with or without activation with TGF- β to collect hPSC-secreted cytokines and growth factors (Fig. 5 A). Incubation of Panc-1 cells with the conditioned media from TGF- β -activated hPSCs showed an induced Panc-1 cell growth and migration compared to the conditioned media from non-activated hPSCs (Fig. 5 B, C). Interestingly, conditioned media collected from LXA4-treated hPSCs showed reduced Panc-1 cell growth and migration (Fig. 5 B, C). For AsPc-1 tumor cells we observed the same trend, with regard to their migration and cell growth, in LXA4-treated hPSCs (Supplementary Fig. 3 A, B) (Supplementary Figure 3 D). Next, we wanted to study the effect of LXA4 on hPSCs in direct contact of Panc-1 cells using a 3D-heterospheroid co-culture system, as shown earlier by us elsewhere ²⁸.

Fig. 5. Effect of LXA4 on hPSC-induced tumor cell migration, proliferation and 3D-heterospheroid formation. Schematic representation of the hPSC conditioned medium experiment (A). Representative images (B) and quantification (C) of Panc-1 tumor cell migration after 24 h of incubation with condition medium collected from untreated, control-treated TGF- β activated hPSCs and 10 nM LXA4-treated TGF- β activated hPSCs. Scale bar = 400 μ m. Panc-1 epithelial tumor cell growth after 48 h of incubation with condition medium collected from untreated, control-treated TGF- β activated hPSCs and 10 nM LXA4-treated TGF- β activated hPSCs (D). Schematic representation of spheroid formation (E). Size of hPSC/Panc-1 3D-heterospheroid formed with control-treated or LXA4-treated hPSCs after 8 days, including representative images of hPSC/Panc-1 3D-heterospheroids formed with control-treated or LXA4-treated hPSCs (F). Scale bar = 100 μ m. Formation rate in percent of hPSC/Panc-1 3D-heterospheroid formed with control-treated or LXA4-treated hPSCs after 4 and 8 days (G). Immunofluorescent staining on 6 μ m thick sections of hPSC/Panc-1 3D-heterospheroid formed with control-treated or LXA4-treated hPSCs for the expression Col1 and nuclei staining (DAPI) (H). Scale bar = 200 μ m. Quantification of Col1 staining divided by

*DAPI staining of hPSC/Panc-1 3D-heterospheroid formed with control-treated or LXA4-treated hPSCs (I). Control contains 0.001% ethanol. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

In order to investigate the effect of LXA4 on 3D-heterospheroid formation and growth, we formed spheroids with hPSC that were treated with LXA4 prior to the experiment (Fig. 5 E). Properly formed spheroids were imaged 8 days after formation using bright-field microscopy and measured for their size (Fig. 5 F). The results showed a significant reduction in spheroid size when hPSCs were treated with LXA4 before spheroid formation when compared to spheroids formed with EtOH-treated hPSCs (Fig. 5 E). Strikingly, spheroids formed with LXA4-treated hPSCs were not yet formed at day 4 after the initiation of spheroid formation, while spheroids containing EtOH-treated hPSCs had already formed (Fig. 5 G). After 8 days the rate of 3D-heterospheroid formation was ~80% for spheroids formed with EtOH-treated hPSCs and ~15% for spheroids formed with LXA4-treated hPSCs (Fig. 5 G). To investigate whether LXA4 does also inhibit hPSC activation in 3D-heterospheroids, we performed immunofluorescent staining on 6 μ m thick sections of hPSC/Panc-1 3D-heterospheroids (Fig. 5 H). We found that spheroids formed with LXA4-treated hPSCs expressed significantly lower levels of Col1 compared to EtOH-treated hPSCs (Fig. 5 H, I). These data signify that LXA4 intervenes into the crosstalk between hPSCs and tumor cells by inhibiting the activation of hPSCs.

4.3.6 LXA4 inhibits the growth of hPSC/Panc-1 3D-heterospheroid

To investigate whether LXA4 also exerts an inhibitory effect on hPSC/Panc-1 3D-heterospheroid growth, we treated the established spheroids (4 days) with either EtOH or LXA4 and followed their growth. Similar to an in vivo tumor model, this 3D model involved the treatment after tumor formation. The spheroids growth was observed over a time period of 12 days. A significant difference in spheroid growth between the LXA4 and control treated group was observed 2 days after the start of the treatment (Fig. 6A, B). Furthermore, the control treated spheroids showed newly formed areas of proliferating cells on their surface, which was not observed in LXA4 treated spheroids (Fig. 6 A). In addition, we examined the effect of LXA4 on the spheroids solely consisting of Panc-1 cells to investigate if LXA4 also inhibits the Panc-1 3D-spheroid growth (Fig. 6 C). No significant decrease in spheroid growth could be observed in the LXA4-treated in comparison to the control Panc-1 3D-spheroids (Fig. 6 C).

Fig. 6. Effect of LXA4 on hPSC/Panc-1 spheroid growth. Representative images (A) and quantification (B) of hPSC/Panc-1 3D-heterospheroid growth after treatment with LXA4 or control and Panc-1 3D-heterospheroids 4 days after initiation of the experiment. Black arrow indicates treatment with LXA4 or control, blue arrows indicate proliferating cells. Growth of Panc-1 3D-spheroids after treatment with LXA4 compared to control treated spheroids (C). Immunofluorescent staining (E) and quantification for the expression of Col1 (F) and nuclei staining (DAPI) (F) in 6 μ m thick section of control and LXA4 treated 3D-heterospheroids after 12 days of spheroid formation. Control contains 0.001% ethanol. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To examine the effect of LXA4 on the activation of hPSCs, we performed immunofluorescent staining on 3D-heterospheroids at day 12. Interestingly, significantly decreased levels of Col1 (Fig. 6 E, F) were found in LXA4-treated

compared to EtOH-treated 3D-hetrospheroids. These data indicate that the reduction in the growth of the 3D-heterospheroids is due to inhibition of hPSC activation and its tumor-inducing effect but not due to a direct effect on the tumor cells.

4.3.7 LXA4 inhibits *in vivo* subcutaneous tumor growth and collagen deposition in mice

To investigate the effect of LXA4 on *in vivo* tumor growth we co-injected hPSCs and Panc-1 into the flank of immunodeficient mice (Fig. 7A).

Fig. 7. Effect of LXA4-treatment on *in vivo* tumor growth and Col1 expression of Panc-1/PSC subcutaneous tumors in mice. Schematic representation of the co-injection (Panc-1 and hPSCs) subcutaneous tumor model (A). The effect of intra tumoral (IT) and intra peritoneal (IP) injection of LXA4 on the tumor growth of co-injection (Panc-1 + PSCs) subcutaneous xenografts in mice (B). Arrow indicate times of injection. The effect of intra tumoral (IT) and intra peritoneal (IP) injection of LXA4 on the body weight of co-injection (Panc-1 + PSCs) subcutaneous xenograft bearing mice (C). Immunostaining (D) and quantification (E) of staining for Col1 in tumors treated IT and IP with LXA4. Scale bar = 200 μ m. Control contains 0.001% ethanol. Data represent

*mean ± SEM for at least 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.*

These tumors are rich in α -SMA and thereby mimic the microenvironment of human PDAC patients (Supplementary Fig. 5). After the tumors were established, we treated the mice with either intratumoral (IT, 4 μ g/kg) or intraperitoneal (IP, 40 μ g/kg) injections of LXA4 or EtOH, twice a week (Fig. 7 B). Both, IT and IP injections of LXA4 significantly inhibited the tumor growth compared to control animals, immediately after the treatment (Fig. 7 B). The difference in tumor size of EtOH-treated and LXA4-treated animals stayed significant until the mice were sacrificed on day 31 after the first injection (Fig. 7 B). LXA-4 treatment showed no significant effects on the body weight of subcutaneous tumor bearing mice (Fig. 7 C). Immunofluorescent staining showed a significant reduction (~60%) of Col1 in both IT and IP treated tumors compared to control tumors (Fig. 7 D, E). Additional stainings for the CAF marker (α -SMA), blood vessel marker (endothelial marker, CD31) did not show significant differences between EtOH- and LXA4-treated tumors, but there was a trend showing the reduction of these markers with LXA4 treatment (Supplementary Fig. 4).

4.4 Discussion

The abundant desmoplastic reaction in PDAC, also known as pancreatic tumor stroma, plays an important role in cancer progression, invasion, metastasis and response to therapy ⁴. Reprogramming of the tumor stroma by dampening the function of hPSCs would be an interesting approach to gain therapeutic benefits for the treatment of cancer. In earlier studies, a natural biomolecule vitamin D has been shown to reverse hPSC activation to improve drug delivery efficiency and prolong survival in animals ⁵. In this study, we explored the therapeutic potential of a highly potent endogenous lipid mediator, LXA4, for the treatment of pancreatic tumors. We showed that LXA4 inhibited the activation of hPSCs into CAF-like myofibroblasts in vitro and thereby inhibited hPSC-induced tumor-promoting effects. In our novel 3D-heterospheroidal co-cultures, LXA4 could inhibit the activation of hPSCs within the 3D microenvironment and reduced spheroid growth. Finally, we showed the therapeutic efficacy of LXA4 in vivo in a co-injection tumor model. This study highlights LXA4 as an interesting biological to be developed for the treatment of pancreatic cancer.

Human PSCs are regarded as the main source of CAFs in the stroma of pancreatic cancer ¹² and as response to activation with TGF- β they acquire a CAF-like

myofibroblast phenotype, as shown previously by us and others ²⁹⁻³¹. LXA4 a highly potent endogenous lipid is known for its anti-inflammatory function and has been shown to inhibit fibrosis in renal mesangial cells, human lung (myo)fibroblasts and experimental renal fibrosis ^{13,23,24}. Here we show that the LXA4-specific surface receptor FPR2 is expressed in hPSCs and highly upregulated in myofibroblast-like activated hPSCs. This result demonstrates that activated hPSCs present a valid target for treatment with LXA4. Our work illustrates for the first time that LXA4 treatment reduces the expression of fibrosis related myofibroblast markers α -SMA and Col1 in TGF- β activated hPSCs in vitro, which was accompanied by a reversal of the elongated, stretched myofibroblast-like phenotype of those cells into their normal phenotype. In addition, LXA4 treatment significantly reduced the TGF- β -induced expression of α -SMA, Col1 α 1, Col3 α 1, PDGFR β , MMP2, Wnt2B, NSDHL, CXCL12, IL6, CTGF, IDI1 and INSIG1, all markers which were previously found to be induced in activated PSCs by transcriptome analysis ^{5,32}. Of special significance are the LXA4 targets Col1 α 1 and Col3 α 1 of the extracellular matrix, limiting chemotherapeutic activity by blocking drug delivery ^{33,34}, and growth factors CTGF and CXCL12, known to potentiate the tumor response to gemcitabine ³⁵ and restore T-cell response ³⁶, respectively. The effects of LXA4 on transcriptional changes in hPSCs were supported by a reduction in hPSC migration upon LXA4 treatment, confirming the significance of LXA4 in controlling hPSC's phenotypic behaviour. These findings illustrate that LXA4 treatment remodels activated hPSCs into their quiescent state by transcriptional remodelling and thereby inhibits the capacity of those cells to support pancreatic tumor growth. Upon activation of hPSCs with TGF- β the Smad 2/3 signalling pathway is activated, resulting in phosphorylation of the Smad 2/3 protein ³⁷. This data demonstrates that TGF- β induced transcriptional and phenotypic changes were controlled by LXA4-induced inhibition of Smad 2/3 phosphorylation, which has earlier been observed by Börgeson et al. in renal rat fibroblasts ³⁸. Previous studies have demonstrated that hPSCs can induce tumor-promoting paracrine effects by secreting mutagenic factors such as growth factors and cytokines to induce tumor progression, invasion and metastasis ^{39,40}. Interestingly, our study shows a modulation of the secretory phenotype of hPSCs after treatment with LXA4, which attenuated hPSC-mediated cell growth and migration of Panc-1 and AsPc-1 tumor cells. These results are in line with previous studies in which hPSCs paracrine effects on tumor cells were reduced by the means of microRNA ²⁹. The inhibitory effect of LXA4 on hPSC activation and hPSC induced tumor promoting effects were not only limited to 2D cell

cultures but also inhibited the formation, growth and ECM-deposition of 3D-heterospheroids formed from hPSCs and Panc-1. These 3D-heterospheroids provide tumor-like characteristics such as a dense, hypoxic core, ECM production and direct crosstalk between stromal and cancer cells. In line with our in vitro data, administration of LXA4 via IT or IP injection into hPSC/Panc-1 subcutaneous tumors in mice reduced Col1 deposition, the major ECM protein within the pancreatic tumor stroma. Finally, the reduced ECM deposition within these tumors, resulted in attenuated tumor progression. In this context, it is important to point out, that ECM degradation resulting in improved intratumoral blood flow and drug delivery has shown survival benefits in pancreatic cancer^{6,41}. Staining for the CAF marker α SMA was reduced in the tumors of mice which obtained IP injections of LXA4 but not in IT treated tumors (Supplementary Figure 4 A), indicating normalization of the CAF phenotype. The reduced α -SMA expression in only IP treated mice points out towards the additional systemic effect of LXA4. However, since the local IT injection reduced the tumor growth, it is clear that the tumor-inhibitory effect of LXA4 is due to a direct effect on the tumor stroma. In addition, LXA4 reduced angiogenesis, a key tumor-promoting mechanism, in tumors of both IT and IP LXA4-treated animals, indicated by the endothelial marker CD31 (Supplementary Figure 4 B).

In conclusion, this study demonstrated for the first time that LXA4 is capable to attenuate tumor growth by inhibiting PSC-induced desmoplasia in pancreatic tumors, by reversing hPSC activation into CAF and modulating hPSC induced tumor promoting effects. Therefore, LXA4 is a potential candidate to be evaluated for the treatment of pancreatic tumor and for its potential to increase the efficacy of chemo- and radiotherapy.

4.5 Financial supports

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4.6 Conflicts of interest

J Prakash is a founder and stakeholder of ScarTec Therapeutics BV, Enschede. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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4.8 Supplementary Data

Supplementary Figure 1. Western Blot showing Smad 2/3 and pSmad 2/3 levels in hPSCs, TGF- β activated hPSCs treated with control or 10 nM LXA4 (A). Quantification showing the ratio of pSmad 2/3 / Smad 2/3 (B). Control contains 0.001 % ethanol. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student *t*-test. **p* < 0.05, ***<0.001.

Supplementary Figure 2. Western Blot showing MMP-2 in control medium, hPSC conditioned medium, conditioned medium from TGF- β activated hPSCs and conditioned medium from TGF- β activated hPSCs treated 10 nM LXA4 (A). Quantification showing the expression of MMP-2 (B). Controls contains 0.001 % ethanol. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. ** $p < 0.01$, *** $p < 0.001$.

Supplementary Figure 3. Representative images (A) and quantification (B) of AsPc-1 tumor cell migration after 24 hours of incubation with condition medium collected from untreated, control-treated TGF- β activated hPSCs and 10 nM LXA4-treated TGF- β activated hPSCs. Scale bar = 400 μ m. AsPc-1 tumor cell growth after 48 hours of incubation with condition medium collected from untreated, control-treated TGF- β activated hPSCs and 10 nM LXA4-treated TGF- β activated hPSCs (C). Controls

contains 0.001 % ethanol. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * <0.05 , ** $p < 0.01$, *** <0.001 .

Supplementary Figure 4. Immunostaining and quantification of staining for α -SMA (A) and CD-31 (B) in tumors treated IT and IP with LXA4. Scale bar = 200 μ m. Control contains 0.001 % ethanol. Scale bar = 400 μ m.

Supplementary Figure 5. Immunostaining for α -SMA in human PDAC and Panc-1 and Panc-1+hPSC subcutaneous mouse tumors. Scale bar = 100 μ m.

Supplementary table 1. Primary and secondary antibodies used for western blot.

Antibody	Source	Dilution
Rabbit polyclonal anti-FPR2	Sigma Aldrich	1:100

Mouse anti- α -SMA	Sigma Aldrich	1:200
Rabbit monoclonal anti-Smad 2/3	Cell Signaling	1:1000
Rabbit monoclonal anti-pSmad 2/3	Cell Signaling	1:1000
Mouse monoclonal anti- β -actin	Sigma Aldrich	1:10000
HRP-conjugated goat anti-rabbit IgG	DAKO	1:2000
HRP-conjugated rabbit anti-goat IgG	DAKO	1:2000
HRP-conjugated rabbit anti-mouse IgG	DAKO	1:1000

Supplementary Table 2. Primary and secondary antibodies used for immunohistochemical staining of cells, spheroids and tumor sections.

Antibody	Source	Dilution
Rabbit monoclonal anti-Smad2/3	Cell Signaling	1:1500
Rabbit monoclonal anti-pSmad2/3	Cell Signaling	1:200
Goat polyclonal anti-Collagen1	Southern Biotech	1:250
Goat polyclonal anti-PECAM-1 (CD31)	Southern Biotech	1:50
Mouse monoclonal anti- α SMA	Sigma Aldrich	1:400
Rabbit anti- α SMA	Thermo Fisher Scientific	1:100
Rabbit monoclonal anti-MMP-2	Cell Signaling	1:1000
HRP-conjugated rabbit anti-goat IgG	DAKO	1:100
HRP-conjugated rabbit anti-mouse IgG	DAKO	1:1000
Alexa Fluor 594 labelled donkey anti-goat	Life-Technologies	1:100
Alexa Fluor 488 labelled donkey anti-rabbit	Life-Technologies	1:100
Alexa Fluor 488 labelled donkey anti-mouse	Life-Technologies	1:100

Chapter 5 - Integrins in wound healing, fibrosis and tumor stroma: High potential targets for therapeutics and drug delivery

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Abstract

Wound healing is a complex process, which ultimately leads to fibrosis if not repaired well. Pathologically very similar to fibrosis is the tumor stroma, found in several solid tumors which are regarded as wounds that do not heal. Integrins are heterodimeric surface receptors which control various physiological cellular functions. Additionally, integrins also sense ECM-induced extracellular changes during pathological events, leading to cellular responses, which influence ECM remodeling. The purpose and scope of this review is to introduce integrins as key targets for therapeutics and drug delivery within the scope of wound healing, fibrosis and the tumor stroma. This review provides a general introduction to the biology of integrins including their types, ligands, means of signaling and interaction with growth factor receptors. Furthermore, we highlight integrins as key targets for therapeutics and drug delivery, based on their biological role, expression pattern within human tissues and at cellular level. Next, therapeutic approaches targeting integrins, with a focus on clinical studies, and targeted drug delivery strategies based on ligands are described.

Graphical abstract

Existing integrin drug targeting ligands, applied for the modification of drug molecules, drug carriers and CAR-T cells (CAR-T), in the context of wound healing, fibrosis or tumor stroma and their respective integrin targets. Dashed lines show potential integrin targets for existing ligands (PHSCNK, PR_b, RGD, RKK12, quinolonic, echistatin) that have not yet been used for targeting the respective cell line. In addition to already targeted integrins, integrins that have not yet been targeted but play a role in the pathology of wound healing, fibrosis or tumor stroma are presented.

5.1 Introduction

Wound healing is a complex process, which if not repaired leads to scar formation so-called fibrosis. Fibrosis, a hallmark of an excessive extracellular matrix (ECM) deposition, accounts for about 45% of mortalities in the modern world ¹. In general, fibrotic diseases are caused by chronic tissue injury, resulting in chronic inflammation and fibrosis which leads to destruction of the normal tissue architecture and ultimately organ failure. Currently, there are no effective treatment opportunities for tissue fibrosis and therefore there is a desperate need for effective anti-fibrotic therapies. In addition, tumors are regarded as “wounds that do not heal” due to extensive fibrosis within tumors. Fibrosis and tumor share a strikingly similar cellular and microenvironmental reactivity. Several tumor types undergo a fibrotic reaction so-called desmoplasia or tumor stroma. Tumor stroma has been shown to strongly support the tumor growth by many means and therefore these tumors are referred to as fibrosis-driven tumors ².

Tissue fibrogenesis is a complex process orchestrated by a bidirectional crosstalk between the different cell types including inflammatory cells, epithelial cells, myofibroblast and ECM in response of the wound healing process ³. Cell fate within fibrotic tissues is profoundly affected by the highly dynamic environment of the pericellular ECM mainly produced by myofibroblasts ⁴. During fibrosis, integrins, a family of transmembrane receptors, mediate various cell-matrix and cell-cell interactions. Integrins facilitate communication between the ECM, non-parenchymal cells including inflammatory cells, fibroblasts, and parenchymal cells, and by these interactions, integrins are directly involved in the initiation and progression of tissue fibrosis ⁴. Therefore, integrins represent highly interesting therapeutic targets.

Within this review, we provide a general introduction of integrins and integrin-mediated signaling, an overview of integrin expression in fibrosis-related cell types, and interaction between integrins and growth factor receptors. The next section describes the role of integrins in wound healing, fibrosis-driven tumor, tumor metastasis and fibrosis. Finally, the last two sections are focused on the novel therapies based on integrin inhibition, including clinical developments, and drug delivery strategies to target integrins.

5.2 Integrin receptors

5.2.1 Integrin heterodimers and ligand specificity

Integrins are a family of heterodimeric cell surface receptors, each consisting of one α and one β subunit. Overall, there are 18 α and 8 β subunits that combine and form heterodimers with 24 different functional integrin receptors identified so far. Each integrin receptor specifically binds to one or more ligands. Their specific ligand binding ability enables cells to connect with its surrounding extracellular matrix (ECM), thereby enabling cell motility and invasion. Integrins possess a physical connection with the inside and the outside of a cell, which allows for bidirectional sensing of signals. With this mechanism, integrins ultimately control cytoskeleton organization, thereby directly affecting essential cellular functions such as cell adhesion, migration, proliferation, survival and differentiation ⁵. The local expression pattern of both integrins and their ligands controls the response of a cell to its microenvironment, as every individual integrin heterodimer is capable to bind multiple ligands and also a ligand may bind to multiple integrin heterodimers ⁵.

In addition to controlling a range of physiological functions, integrins also sense ECM-induced extracellular changes during pathological events such as fibrosis, cancer and wound healing, leading to cellular responses, which influence ECM remodeling ⁵. Next to binding ECM components, integrins are also capable of participating in cell-cell adhesions, for which they bind to counter receptors on adjacent cells such as ADAMs (A Disintegrins And Matrix metalloproteinases (MMPs)), thereby promoting matrix remodeling ⁶; as well as immunoglobulin-type receptors such as intracellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs) which are expressed on leukocytes and endothelial cells ⁷.

Integrins can be classified into five different integrin subfamilies (Table 1) ⁶. Integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$, belong to the $\beta 1$ containing collagen receptors ⁶. The integrins $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha \nu\beta 1$, $\alpha \nu\beta 3$, $\alpha \nu\beta 5$, $\alpha \nu\beta 6$, $\alpha \nu\beta 8$ and $\alpha \text{IIb}\beta 1$, belong to the RGD (arginine-glycine-aspartic acid)-binding integrins capable of binding to the ECM and plasma proteins such as fibronectin, vitronectin, fibrinogen and thrombospondin ^{6,8}. The integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$ and $\alpha 6\beta 4$ are laminin receptors that mediate cell adhesion to the basement membranes of various tissues ⁶. The $\alpha 4\beta 1$, $\alpha 9\beta 1$, $\alpha 4\beta 7$ integrin family also binds to fibronectin but in a RGD-independent manner via the adhesive sequences EILDV and REDV manner ^{6,8}. Additionally, integrin $\alpha 4\beta 1$ and $\alpha 4\beta 7$ are also able counter

receptors in other cells e.g. intercellular adhesion molecules ⁶. Integrins α D β 2, α L β 2, α M β 2, α X β 2 and α E β 2 belong to the leukocyte integrin subgroup, binding to receptors such as intracellular adhesion molecules (ICAMs) and plasma proteins such as complement component C3b and C4b⁶.

Table 1. Integrin heterodimer subfamilies comprised of 24 integrin receptors and their respective ligands.

Integrin receptor subfamilies	Integrin Type
Collagen receptors	α 1 β 1, α 2 β 1, α 10 β 1, α 11 β 1
Fibronectin, vitronectin, fibrinogen and thrombospondin receptors (RGD binding)	α 5 β 1, α 8 β 1, α v β 1, α v β 3, α v β 5, α v β 6, α v β 8, α IIb β 1
Laminin receptors	α 3 β 1, α 6 β 1, α 7 β 1, α 6 β 4
Fibronectin receptors (non-RGD binding)	α 4 β 1, α 9 β 1, α 4 β 7
Leukocyte receptors	α D β 2, α L β 2, α M β 2, α X β 2, α E β 2, α 4 β 1, α 4 β 7

5.2.2 Integrin signaling

During interactions of integrins with ligands of the surrounding ECM, integrins undergo a conformational change and cluster in the plane of the cell-membrane ⁸. This change in conformation activates integrins to a high avidity state. Within this state, integrins recruit various signaling and adaptor molecules to form focal adhesions ⁸. The composition of these focal adhesions varies dependent on whether these contacts are formed in a two-dimensional or three-dimensional environment ⁹. While lacking kinase activity by themselves, clustered integrins are capable of recruiting and activating kinases such as focal adhesion kinase (FAK), Src family kinases (SFKs) and scaffold molecules such as p130CRK-associated substrate (p130CAS or BCAR1) ⁸.

Next to activating kinases, integrins can also connect ECM to the actin cytoskeleton by recruiting proteins including talin, paxillin, α -actinin, tensin and vinculin ⁸. Furthermore, several scaffolding and signaling functions required for integrin-mediated effects on cell migration and survival are controlled by the integrin-linked kinase (ILK), PINCH and parvin. The ILK-PINCH-parvin (IPP) ternary complex functions as an essential signaling platform that regulates various scaffolding and signaling functions which enable integrin-mediated effects on cell migration and survival ¹⁰. Moreover, integrins function in tumour

cells might be regulated by integrin recruitment to microdomains by tetraspanin⁸. Regulation and activation of microdomains and other focal adhesion proteins influence cell adhesion and migration on the ECM. Many of these molecules are investigated as promising therapeutic targets⁸. In some cases, integrin function is based on its ligand binding affinity⁸. The affinity and activation of an integrin receptor can be induced by ligand-mediated integrin clustering or increased intracellular signaling driven by molecules such as GTPase RAP1A¹¹. Therefore, signaling which is induced by oncogenes or growth factor receptors might influence integrin affinity and function⁸.

5.2.3 Integrins and growth factor receptor interaction

Integrins and growth factor receptors play an important role for signal integration¹². Crosstalk between integrins and growth factor receptors have been described for the TGF β receptor, epidermal growth factor receptor (EGFR), Met receptor (belongs to HGFR superfamily, hepatocyte growth factor receptor), platelet derived growth factor receptor (PDGFR), insulin receptor and vascular endothelial growth factor receptor (VEGFR)¹². There are various different classes of signal integration between different integrins and growth factor receptors¹³. In the following, we describe the five classes of signal coordination (i) concomitant signaling, (ii) collaborative signaling, (iii) direct activation and (iv) amplification of signaling and (v) negative regulation (Figure 1)¹³.

(i) Concomitant signaling:

During concomitant signaling, integrins together with growth factor receptors signal independently to trigger the same signaling molecule (Figure 1). Pathways which are affected by concomitant signaling include Ras-MAPK (mitogen-activated protein kinase), PI3K-Akt (PI3K (phosphatidylinositol 3-kinase) and Akt (Protein Kinase B), and Rho¹²⁻¹⁵. As an example Akt can be activated by integrins and growth factor receptors in a PI3K-dependent manner via distinct mechanisms¹³. Phosphorylation of Akt at Ser473 and Thr308 and of Akt kinase activity is induced by integrin β 1 independently of epithelial growth factor receptor signaling, while epithelial growth factor induces Akt activity via the Fak and Src independent of cell adhesion¹³.

(ii) Collaborative signaling:

Collaborative signaling is very similar to concomitant signaling in the way that cells require integrin-mediated adhesion to proceed through the cell cycle and respond to growth factors since growth factor receptor signaling is inefficient in the absence of cell adhesion¹³. During collaborative signaling, integrin receptors create a permissive environment in which growth factor receptors can interact with downstream signaling molecules (Figure 1)¹⁶. The difference between concomitant and collaborative signaling is that during collaborative signaling the receptor signals are spatially and temporally controlled while in concomitant both integrin and growth factor receptor work independently¹³. An example for this type of signaling is that cells which only express Met in the absence of integrin $\alpha 6 \beta 4$ no-longer respond to HGF showing the collaboration between integrin $\alpha 6 \beta 4$ and the Met receptor (HGFR)¹⁷.

(iii) Direct activation:

During direct activation of growth factor receptors, integrins induce growth factor phosphorylation in absence of the corresponding growth factor (Figure 1). This ligand-independent activation process has been shown for the growth factor receptors EGFR, Ron (Recepteur d'Origine nantais, member of HGFR superfamily), VEGFR, IGFR (insulin-like growth factor receptor) and PDGFR¹⁸⁻²⁵. An example for direct activation is the ability of integrin $\alpha v \beta 3$ to activate VEGFR-2, IGFR-1 and PDGFR in a growth factor independent manner²¹⁻²⁶. Additionally, in macrophages integrin $\beta 1$ is associated with Ron and the adhesion to collagen or fibronectin results in the phosphorylation of Ron in a Src-dependent manner and binding of integrin $\alpha 5 \beta 1$ by fibronectin induces activation of Met^{20,26}.

(iv) Amplification of signaling:

Another form of crosstalk between integrins and growth factor receptors is amplification of signaling. It is based on the ability of growth factors to activate signaling by binding to their corresponding growth factor receptors which can then increase the expression of integrins¹³. This amplification process has for example been shown for HGF, increasing the expression of integrin $\alpha 2$ and $\alpha 3$ ^{27,28}, which could contribute to the amplification of Met signaling in response to HGF via the FAK-Src axis¹³.

(v) Negative regulation:

As described above, integrins mostly function as positive regulators of growth factor receptor signaling. However, integrin interaction with the ECM can also inhibit growth factor receptor signaling through phosphatase activation and recruitment of, for example, T cell protein tyrosine phosphatase (TCPTP) (Figure 1)¹³. In renal fibrosis, it has been shown that Integrin $\alpha 1\beta 1$ reduces Smad-dependent profibrotic signaling in kidney collecting duct derived cells by TCPTP-mediated dephosphorylation of TGF β R2 (a variant of the TGF β receptors II)²⁹.

Figure 1. Schematic representation and description of the distinct mechanisms by which integrins and growth factor receptors regulate the activation of signaling pathways within the cell. During concomitant signaling, integrins together with growth factor receptors, signal independently to trigger the same signaling molecule. Thereby, integrins gather a platform of signaling proteins, which facilitate growth factor receptors signaling. During direct activation, integrin activate growth factor receptors signaling independent of growth factors. In collaborative signaling, integrin receptors create an permissive environment which enables growth factor receptors to interact with downstream signaling molecules. Growth factor receptor signaling trigger increased integrin expression, which can further induce growth factor receptor signaling. Integrin interaction with the ECM can cause negative regulation of growth factor receptor signaling through phosphatase activation and recruitment of, for example, T cell protein tyrosine phosphatase (TCPTP). Integrins that are involved in the different signaling

mechanism are stated as examples. The cooperation between integrins and growth factor receptors has been reviewed in more detail by Ivaska et. al. ¹³.

5.2.4 Integrin and growth factor receptor interactions

The following are some examples showing interactions between integrins and growth factor receptors in relation to fibrosis and cancer. Growing evidence suggests that the progression of fibrosis is affected by signaling between integrins, growth factor receptors and cytokine or chemokine receptors. Next to cell adhesion, migration, invasion and survival, integrin crosstalk also affect the host response to fibrosis driven diseases ⁸.

(i) Interaction with TGFβR:

Transforming growth factor beta 1 (TGFβ1), in its secreted form, is one of the main pro-fibrotic cytokine and regulator of fibrosis in multiple organs ³. Most of the pro-fibrogenic TGFβ1 is secreted and bound to the ECM in its latent form ³. Conversion of latent TGFβ1 into its active form is an important step that regulates TGFβ1 activity ³. αv integrins are known for their ability to activate latent TGFβ1 ⁴. Evidence for the interaction between TGFβ and integrins came from the structural analysis of the molecule by Ruoslahti and Pierbacter *et al.* ³⁰, which suggested that TGFβ1 & TGFβ3 bind to integrins based on their linear sequence of arginine, glycine and aspartic acid (RGD), which is known to be crucial for the interaction of many integrins with their respective ligands. The integrins αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 were identified to bind to the RGD-sequence of the latency associated peptide (LAP) of TGFβ1 and TGFβ3, and are capable to activate latent TGFβ ³¹⁻³⁵. During pulmonary inflammation and fibrosis, TGFβ1 activation is regulated by integrin αvβ6 ³⁶. Integrin αvβ6 activates TGFβ by inducing a conformational change in the integrin αvβ6-bound latent TGFβ complex which then presents active TGFβ to its receptor on adjacent cells via cell-cell contact ^{31,36}. Marsh *et al.* ³⁷ showed that integrin αvβ6 dependent activation of TGFβ resulted in the differentiation of human fibroblasts into tumor stroma-associated myofibroblasts. Myofibroblasts are capable to activate TGFβ1 from self-generated deposits in the ECM by means of αvβ5 integrins which transmits the highly contractile forces of these cells to the latent complex of TGFβ1. Additionally, integrin αvβ8 has been shown to activate TGFβ by presenting the latent TGFβ complex to metalloproteinases that cleave the complex, resulting in the release of free TGFβ into the extracellular milieu ³⁶.

Integrin $\alpha\beta3$ is known to induce EMT in mammary epithelial cells by cooperating with TGF β via Src-dependent phosphorylation of TGF β receptor type 2³⁸. Integrin $\beta3$ deficiency in mice was shown to correlate with elevated levels of TGF β receptor 1 and 2, reduced levels of Smad 3, sustained nuclear localization of Smad 2 & 4 and TGF β 1-mediated fibroblast migration³⁹. These data indicate that integrin $\alpha\beta3$ is expressed on platelets, macrophages, endothelial cells and fibroblasts during wound repair and is capable of repressing TGF β 1-mediated signaling³⁹. Increased expression of $\alpha\beta5$ in fibroblasts increases their responsiveness to TGF β 1 by recruiting latent TGF β 1 on the cell surface and stimulating the interaction between $\alpha\beta5$ and the TGF β receptor⁴⁰.

(ii) Interaction with VEGFR2:

During angiogenesis, endothelial cells express integrin $\alpha\beta5$ that interacts with VEGF receptor 2 (VEGFR2) to promote VEGF-induced angiogenesis via the Ras-ERK pathway^{41,42}. Additionally, integrin $\alpha\beta5$ in cooperation with VEGFR2 causes inflammatory mediators (e.g. tumor necrosis factor) induced resistance of endothelial cells to extrinsic apoptosis, via Src-dependent phosphorylation of Raf Tyr340 and Tyr341⁵. Mice with genetic knockout of integrin $\beta3$ showed an abnormal endothelial cell morphology which was associated with increased VEGF signaling⁴³. Integrins also play a major role in the control of neo-vascularization in wound healing, where they act as co-receptors for the growth factor-receptors VEGF and angiopoietin, and support the assembly of vascular membranes⁶.

(iii) Interaction with FGFR:

In endothelial cells, cross-talk between integrin $\alpha\beta3$ and fibroblast growth factor receptor (FGFR) was found to induce angiogenesis downstream of FGF binding⁴². Evidences support that FGFR and integrin $\alpha\beta3$ cooperate to increase the phosphorylation of Raf Ser338 and Ser 339 through PAK (p21-Activated Protein Kinases) pathway, resulting in Raf-ASK1 complex formation in mitochondria, thereby inhibiting the intrinsic apoptosis pathway⁸. Furthermore, knockdown studies in mice have shown that integrin $\beta4$ expression correlates with decreased FGF-induced angiogenesis and reduced tumor size via P-ERK and NF-kappaB signaling pathways⁴⁴.

Integrin $\alpha1\beta1$ negatively regulates epidermal growth factor (EGF) receptor-mediated Rac activation, thereby reducing the production of reactive oxygen species in mesangial cells resulting in attenuation of fibrogenesis in mice⁴⁵. Additionally, other studies have shown that integrin $\alpha1\beta1$ also regulates

activation of TCPTP, resulting in the inhibition of EGFR and VEGFR2 signaling^{46,47}.

(iv) Interaction with other growth factor receptors:

Integrin $\beta 4$ was found to functionally collaborate with the Met tyrosine kinase receptor for hepatocyte growth factor (HGF) resulting in increased fibroblast transformation and tumorigenic potential⁴⁸. The expression of insulin-like growth factor 2 (IGF-2) has shown to be stimulated by the expression of integrin subunit $\alpha 11\beta 1$ in stromal fibroblast of non-small-cell lung carcinoma⁴⁹.

In primary cultures of hepatic stellate cells, the liver fibrosis promoting connective tissue growth factor, CTGF (or CCN2), was shown to regulate the expression of integrins on hepatic stellate cells (HSCs) and additionally facilitate HSCs adhesion via binding to integrin $\alpha 5\beta 1$, which interacts cooperatively with heparin sulfate proteoglycans or fibronectin⁵⁰.

5.3 Integrins as key targets in wound healing, fibrosis and tumor stroma

The following section describes the cell type specific expression of integrins (summarized in Figure 2) as well as their functional role in wound healing, tumor stroma, metastasis and kidney, liver and lung fibrosis (Table 2).

5.3.1 Wound healing

Wound healing is a common repair process after an injury to an organ. We describe the repair of cutaneous wounds in this section. Wound healing is based on the collective efforts of soluble mediators, blood cells, extracellular matrix and parenchymal cells⁵¹. This process consists of three timely overlapping stages namely, inflammation, tissue formation, and tissue remodeling⁵¹. During the innate inflammation, immune cells including neutrophils and granulocytes in the early phase and then macrophages, lymphocytes and mast cells are recruited into the wound. These immune cells release cytokines and chemokines that recruit epithelial cells and fibroblasts to the wound edge⁶. Thereafter, epithelial cells start to stretch into the wound bed, followed by proliferating keratinocytes, which seed more cells into the wound site during re-epithelialization⁶. Parallel to re-epithelialization, granulation tissue formation is initiated which is closely associated with wound angiogenesis⁶. During this process, epithelial cells are recruited into the wound by cytokines and create the granulation tissue together

with myofibroblasts and pericytes⁶. Myofibroblasts facilitate wound contraction and closure which is followed by tissue remodeling, during which myofibroblasts degrade, remodel and reorganize the ECM⁶. During the complex wound healing process, cells bind to ECM molecules within the wound via their integrin receptors resulting in integrin's functional activation or induced expression. Integrin receptors with a functional role in wound healing are listed in table 2.

Integrin $\beta 1$ as an important integrin because it is a subunit in many different heterodimers. Integrin $\beta 1$ deficiency in mouse fibroblasts, correlates with reduced expression of α -smooth muscle actin (α -SMA), CCN2/CTGF and collagen I, and is accompanied by a reduced ability to activate latent TGF β , thereby inhibiting differentiation of fibroblasts into myofibroblasts, resulting in delayed wound closure and reduced formation of granulation tissue⁵². Integrin $\alpha v \beta 3$ has shown to be inhibitory to the fibroblast infiltration into the wound clot. Mice deficient of integrin $\beta 3$ showed accelerated re-epithelialization, which is associated with enhanced TGF β signaling and dermal fibroblast infiltration into wounds³⁹. Integrin $\alpha 1 \beta 1$ is expressed on cells of the basement membrane including vascular, visceral, endothelial, smooth muscle cells and pericytes, and on cells of the connective tissue including fibroblasts, chondrocytes, mesenchymal stem cells and circulating white blood cells⁵³. The collagens that bind integrin $\alpha 1 \beta 1$ include collagen I, III, IV, XIII, XVI⁵³. Modulating the collagen-binding integrin activity could therefore also be an interesting approach to improve the healing of chronic wounds.

Integrin $\alpha 2 \beta 1$ is expressed on keratinocytes, epithelial cells and endothelial cells which are in contact with the basement membrane and on fibroblasts, T-cells, myeloid cells, megakaryocytes and platelets which are in contact with matrices rich in collagen I⁵⁴. Integrin $\alpha 2 \beta 1$ specifically binds to collagen I, III, IV, V, XI, XVI and XXIII⁵⁴. Next to collagens, integrin $\alpha 2 \beta 1$ also binds to the proteoglycans, biglycan, lumican, fibromodulin and decorin, and a proteolytic fragment derived from perlecan called endorepellin⁵⁴. It has been shown that binding of fibroblasts to collagen within 3D-matrices activates p38 α MAP kinase pathway and results in increased integrin $\alpha 2 \beta 1$ -dependent collagenase-3 (MMP13) synthesis^{54,55}.

Mice deficient in $\alpha 1$ and $\alpha 2$ demonstrated minor changes in their ability to remodel granulation tissue ECM, this is likely because other collagen-binding integrins compensate for most of their function, including MMP expression and

collagen fibrillogenesis⁶. Integrin $\alpha 3\beta 1$ is expressed on basal cells of epidermis and other epithelia⁵⁶. Conditional knockout of integrin $\alpha 3\beta 1$ in the epidermis of mice (keratinocytes) resulted in impaired angiogenesis within wounds, which is correlated with reduced expression of the angiogenesis promoting mitogen-regulated protein 3 (MRP3)⁵⁷. These findings suggest a role of $\alpha 3\beta 1$ in promoting wound angiogenesis through MRP3-mediated crosstalk from epidermal to endothelial cells⁵⁷.

During wound healing, fibronectin (Fn) is activated and assembled into a fibrillary structure Fn matrix that is known to be promoted by integrin $\alpha 5\beta 1$. Fibroblasts adhesion to the provisional matrix (composed of Fn and fibrin) via integrin $\alpha 5\beta 1$ in the initial stage of wound healing is enhanced when dermatopontin (a dermal ECM protein) co-localizes with fibrin and fibronectin in the wound clots⁵⁸. Additionally, the interaction of $\alpha 5\beta 1$ with fibronectin has shown to contribute to T-Cell lymphokine-driven keratinocyte proliferation next to facilitating matrix adhesion and motility⁵⁹. It has been speculated that in fibroblasts, integrin $\alpha 5\beta 1$ plays an important role *in vivo* during invasion of connective tissue cells into the wound clot and their migration in the fibrin-fibronectin-containing 3D wound environment⁶. Fibroblasts in the granulation tissue have a reduced ability to bind fibronectin via integrin $\alpha 5\beta 1$ which might allow them to migrate in the early fibronectin-rich granulation tissue matrix⁶⁰. Blocking of integrin $\alpha 5$ with antibodies *in vitro* in human oral mucosa and dermal fibroblasts were capable of blocking TGF β -induced expression of α -SMA⁶¹. This finding implies that novel therapeutic approaches targeting integrin $\alpha 5$ could present a strategy to inhibit α -SMA positive myofibroblasts which are closely associated with scar formation and various other pathological disorders.

In literature, the increased integrin $\alpha 9\beta 1$ expression is shown to induce retarded wound re-epithelialization, as shown in $\alpha 9\beta 1$ -deficient mice⁶. Furthermore, integrin $\alpha 9\beta 1$ controls proliferation of keratinocytes and dermal fibroblast by interacting with elastic microfibril interface-located protein 1 (EMILIN1)⁶². Additionally, blocking of integrin $\alpha 9\beta 1$ on integrin-positive dermal fibroblasts with a specific antibody inhibited the formation of granulation tissue in cutaneous wound healing, showing that integrin $\alpha 9\beta 1$ is involved in the formation of granulation tissue by regulating the migration and adhesion of dermal fibroblasts in excisional wounds⁶³.

Integrin $\alpha 11\beta 1$ expression has also been shown to be expressed restrictively to a subset of fibroblasts and mesenchymal stem cells *in vivo*⁶⁴ and is the main

collagen receptor on dermal fibroblasts, contributing to collagen remodeling in a TGF β -dependent manner⁶⁵. Zweers *et al.* were the first to demonstrate the role for integrin $\alpha 11\beta 1$ in dermal wound healing, in which $\alpha 11\beta 1$ is strongly induced in mice after inflicting excisional wounds⁶⁶. Dermal wounds in integrin $\alpha 11\beta 1$ deficient mice showed a reduction in granulation tissue formation and wound strength 7 days after excisional wound infliction, which is attributed to a defect in myofibroblasts differentiation, indicating $\alpha 11\beta 1$ -dependent collagen remodeling within granulation tissue⁶⁵. Next to its role in collagen remodeling, integrin $\alpha 11\beta 1$, similar to integrin $\alpha 5\beta 1$, is involved in myofibroblast differentiation and granulation tissue formation, as a response to injury, and thereby contributes to scar formation. This implies integrin $\alpha 11\beta 1$ as a potential therapeutic target and it would be of very high interest to investigate the effects of therapeutic blocking of this integrin in the context of myofibroblast differentiation during scar formation and pathological fibrosis in general.

In addition to fibroblasts and epithelial cells, integrins play a key role in immune cells such as neutrophils, monocytes and certain lymphocytes. Integrin $\alpha M\beta 2$ is a leukocyte receptor which is involved in immune cell recruitment and the activation of inflammatory responses during wound healing⁶⁷. Integrin $\alpha M\beta 2$ is capable of engaging various ligands including ECM proteins, counter receptors as intracellular adhesion molecule 1 (ICAM-1) and coagulation and complement products. Ligand binding by integrin $\alpha M\beta 2$ affects leukocyte adhesion and activation⁶⁷. Knockout of integrin $\alpha M\beta 2$ in mice has been shown to be correlated with a delay in wound re-epithelialization and granulation tissue formation but did not affect monocyte migration into the wound⁶⁷.

5.3.2 Fibrosis

In general, fibrosis is a response to organ injury and progressive fibrosis can lead to major organ failure and ultimately lethality^{3,68}. Organ fibrosis is characterized by a complex interplay between inflammatory, epithelial, myofibroblast, and excessive ECM production and deposition^{3,68}. The highly dynamic pericellular ECM of the fibrotic tissue exerts profound influences on the behavior of the surrounding cells³. Many of the main cell-cell and cell-matrix interactions that regulate fibrosis are mediated by integrins³. The expression and function of integrins in kidney, liver and lung fibrosis are described in the following section and is listed in table 2.

5.3.2.1 Kidney fibrosis

Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, the major collagen binding receptors, and laminin receptors $\alpha 3\beta 1$ and $\alpha 6\beta 1$ are highly expressed in the healthy kidney ⁶⁹. Integrin $\alpha 1\beta 1$ binds to collagen IV, and deletion or inhibition of $\alpha 1\beta 1$ exacerbates glomerulosclerosis suggesting that activation of $\alpha 1\beta 1$ integrin might be beneficial for renal injury ⁷⁰. In contrast to Integrin $\alpha 1\beta 1$, integrin $\alpha 2\beta 1$ is a positive regulator of collagen synthesis and reactive oxygen species production. Studies propose that Integrin $\alpha 2\beta 1$ induces glomerular fibrosis and absence of $\alpha 2\beta 1$ delays kidney fibrosis and glomerular injury in experimental models for kidney disease ^{71,72}. Knockdown of the discoidin domain receptor 1 (DDR1) and integrin $\alpha 2\beta 1$ delays the maturation of the glomerular basement membrane, which causes renal fibrosis in the Col4A3^{-/-} mice, a mouse model of Alport syndrome ⁷². Additionally, in Col4A3^{-/-} Alport mice with impaired glomerular basement membrane, maturation loss of integrin $\alpha 2\beta 1$ delays kidney fibrosis ⁷². An additional collagen binding integrin, integrin subunit $\alpha 11$ is specifically localized on myofibroblasts in UUO kidneys in mice and human fibrotic kidneys was found to be crucial for the regulation of the myofibroblast phenotype ⁷³. Moreover, its expression was significantly induced at an interstitial fibrosis and tubular atrophy score of 3, when compared to score 0-2 ⁷³. In Alport mice with a conditional knockdown of integrin $\beta 6$, renal fibrosis was inhibited ⁷⁴. Furthermore, knockdown of integrin $\beta 6$ partly or completely protects mice from tubulointerstitial fibrosis induced by kidney obstruction ⁶⁸.

The expression of integrin $\alpha \nu \beta 1$, $\alpha \nu \beta 3$ and $\alpha \nu \beta 5$ was also identified on renal fibroblasts and blockade of $\alpha \nu \beta 1$ prevented the activation of latent TGF $\beta 1$ through direct binding by fibroblasts ⁷⁵.

While in the healthy kidney, integrin $\alpha 8$ is only expressed in mesangial cells and vascular smooth muscle cells, de-novo expression of integrin $\alpha 8$ was found on interstitial fibroblasts and tubular epithelial cells undergoing de-differentiation in tubulointerstitial fibrosis induced by the unilateral ureteral obstruction model ⁷⁶. Furthermore, studies in mice with knockdown of integrin $\alpha 8$ revealed that underexpression of $\alpha 8$ did not inhibit tubulointerstitial fibrosis, but increased tubulointerstitium damage compared to wild type mice ⁷⁶. Therefore, integrin $\alpha 8$ targeting does not seem to be a useful anti-fibrotic therapeutic target.

Moreover, integrin $\alpha 3$ has shown to induce kidney damage attributed to loss of E-cadherin induced by integrin $\alpha 3$ -dependent Src/p- β -catenin-Y654/p-Smad2-mediated up-regulation of integrin-linked kinase ⁷⁷.

In summary, integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha \nu\beta 3$, $\alpha 3$ and $\alpha 8$ have been identified to play a role in kidney fibrosis. Inhibiting integrin $\alpha 2\beta 1$ and $\alpha \nu\beta 3$ in kidney fibrosis seems have high potential as a potential therapy, while induction of integrin $\alpha 1\beta 1$ and integrin $\alpha 3$, via e.g. an RNAi approach, also seems to have therapeutic potential.

5.3.2.2 Liver fibrosis

In healthy liver, different integrins are expressed at different cell types controlling specific functions to maintain homeostasis. Vascular endothelium expresses many integrins such as $\alpha 1$, 2, 3, 4, 5 and 6; bile duct epithelium express integrin $\alpha 2$, 3, 5 and 6; stroma of the connective tissue integrin $\alpha 1$ and 2; hepatocytes integrin $\alpha 1$ and 5; sinusoidal lining cells integrin $\alpha 1$, 2, and 5; and mononuclear cells integrin $\alpha 4$ ⁷⁸. During liver pathogenesis, the altered expression levels and *de novo* expression of integrins have been reported in preclinical and clinical studies. Neijjari *et al.* performed a clinical study including 94 patients with chronic hepatitis C, in which the expression of integrin $\beta 1$, $\alpha 1$, $\alpha 5$ and $\alpha 6$ was significantly upregulated and showed correlation with the stage of fibrosis ⁷⁹. In a different study, integrin $\beta 6$ was shown to correlate with the stage of fibrosis in the livers of patients with end-stage liver disease, including chronic hepatitis B & C, primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) ⁸⁰. Mice with integrin $\beta 6$ knockdown or blocking antibody to $\alpha \nu\beta 6$ significantly decreased acute biliary fibrosis after bile duct ligation ⁶⁸. In patients with alcoholic liver disease, integrin $\beta 1$ was significantly overexpressed on T lymphocytes (and/or hepatocytes) when compared with healthy patients ^{81,82}. In liver cirrhosis, integrin αL , αM , αX and $\alpha 4$ expression levels on peripheral blood leukocytes was positively correlated with liver failure ⁸³. During cholestasis, the hepatocytes show *de novo* expression of integrin $\alpha 3$ and $\alpha 6$, indicating that they undergo a phenotypic switch from hepatocytes to bile duct epithelium ⁷⁸. In PBC, integrin $\alpha L\beta 2$ (lymphocyte function associated antigen 1 (LFA-1)) and $\alpha 4\beta 1$ (very late antigen 1 (VLA-4)) were found to be expressed on infiltrating lymphocytes, while control livers showed no or weak expression of these integrins ⁸⁴.

In addition to these clinical studies, integrin $\alpha \nu\beta 6$ was found to be upregulated in rotavirus-induced biliary atresia, resulting in liver fibrosis ⁸⁵. Rats with a chronic alcohol intoxication showed an induced expression of integrin $\beta 2$ on neutrophils, which increases their migration (most probably mediated by osteopontin via $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrins ⁸⁶) and Kupffer cells mediated release of chemotactic cytokines and growth factors ⁸⁷. Moreover, in PBC, integrin αL

(CD11a) was found to be expressed on T lymphocytes but is absent in chronic hepatitis C or healthy patients, indicating a role of CD4⁺ integrin α L expressing lymphocytes in Th-1 predominance and might thereby indirectly promote fibrosis⁸⁸. Mice with an integrin β 2 knockout showed reduction in hepatic necrosis, decreased number of intrahepatic neutrophils and plasma transaminase activity during acute and chronic cholestatic liver injury^{89,90}. In chronic liver inflammation and fibrogenesis, integrin β 1 activated by vascular adhesion protein-1 and CX₃ chemokine receptor 1 has been proposed to be responsible for the recruitment of CD16(+) monocytes into the liver⁹¹. Activation of HSCs increases the expression of integrin α 5 β 1, and α 5 β 1/ECM crosstalk induced collagen production via changes in the cytoskeletal organization, and activation of Src kinases and ERK/JNK signaling molecule families⁹². Expression of integrin α 8 β 1 is induced in activated HSCs following bile duct ligation or CCl₄ induced hepatic injury in rats⁹³. Genetic knockdown (Pdgfrb-Cre) of integrin α v protected mice from CCL4-induced liver fibrosis and was also protective in pulmonary and renal fibrosis⁹⁴. Very recently, Bansal *et al.*⁷³, have identified integrin subunit α 11 as a major regulator in the activation of HSCs into myofibroblasts. Knockdown of integrin subunit α 11 in HSCs inhibited their differentiation and functionality in response to TGF- β ⁷³. Integrin subunit α 11 expression was found to be regulated by the hedgehog pathway and inhibition of hedgehog led to inhibition of HSC induced fibrosis in mice. This work highlights integrin subunit α 11 as a highly promising therapeutic target in liver fibrosis⁷³.

5.3.2.3 Lung fibrosis

Integrin α v β 6, a receptor for the ECM proteins fibronectin⁹⁵ and tenascin C³⁶ is minimally expressed in aveolar epithelial tissues but is highly induced upon lung injury, resulting in lung fibrosis⁹⁶. Integrin α v β 6 has been demonstrated to be overexpressed in the epithelium of lung sclerosis and pulmonary fibrosis⁶⁸. Genetic knockdown of integrin β 6 in mice was introduced to attenuate bleomycin-induced pulmonary fibrosis and radiation induced pulmonary fibrosis^{31,68}. Recently, integrin α 6 β 1, upregulated in fibrotic lung myfibroblasts, was identified as a mechanosensor for matrix stiffness,⁹⁷. Upon sensing matrix stiffening during pulmonary fibrosis α 6 β 1 mediates MMP-2 dependent pericellular proteolysis of basement membrane collagen IV, thereby regulating invasion of myofibroblasts⁹⁷. The expression of integrin α v β 8 is highly expressed in the airways of chronic obstructive pulmonary disease (COPD) patients and correlates with the severity of the obstruction, and COPD fibroblasts show increased pro-fibrogenic differentiation upon α v β 8 mediated TGF β 1

activation^{98,99}. Knockdown of $\alpha\text{v}\beta 8$ in murine lung fibroblasts reduced TGF β -activation in these cells¹⁰⁰. Deletion of $\alpha\text{v}\beta 8$ in lung fibroblasts resulted in inhibition of airway fibrosis in IL-1 β and ovalbumin-induced mouse models¹⁰⁰. Additionally, the authors demonstrated that IL-1 β increased $\alpha\text{v}\beta 8$ -dependent TGF β activation, collagen expression and pro-inflammatory gene expression in human COPD compared to normal human lung fibroblasts¹⁰⁰. In patients with idiopathic pulmonary fibrosis, integrin $\alpha\text{v}\beta 5$ causes TGF β -mediated fibrosis and co-localizes with PAR1 (Protease-activated receptors) and the myofibroblast marker αSMA ¹⁰¹. This process is inhibited by the blockade of integrin $\alpha\text{v}\beta 5$ in mice¹⁰¹. Kim *et al.* showed that in idiopathic pulmonary fibrosis (IPF), alveolar epithelial cells undergo extracellular matrix triggered EMT, thereby turning into differentiating fibroblasts¹⁰². In an IPF mouse model with a lung specific deletion of integrin $\alpha 3\beta 1$ reduced accumulation of myofibroblasts, collagen and genes associated with EMT were observed^{103,104}. Integrin subunit $\alpha 11$ was in an additional study found to be significantly induced in the fibrotic lungs from patients with IPF where it co-localizes with $\alpha\text{-SMA}$ -positive myofibroblasts⁷³. In addition, the expression of integrin subunit $\alpha 11$ correlated concomitantly with the expression of various fibrotic parameters in the lungs of patients with IPF⁷³.

5.3.3 Tumor stroma

The tumor stroma consists of non-cancerous cells including cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), pericytes, endothelial cells, and infiltrating immune cells¹⁰⁵. More recently, stromal cells have been identified as drivers of tumorigenesis, promoting tumor growth, angiogenesis, invasion and metastasis¹⁰⁵. Integrins that were found to be expressed in these cell types and their functional role in the stroma is discussed in the following section and listed in table 2.

Integrin $\alpha 11\beta 1$ and $\alpha 5\beta 1$ have been reported to be the main integrins expressed on fibroblasts within the tumor stroma^{106,107}. Integrin $\alpha 11\beta 1$ was found to be induced in a mechano-sensitive manner and contributes to TGF β -dependent myofibroblasts differentiation *in vitro*¹⁰⁸. A role for integrin $\alpha 11\beta 1$ in tumorigenesis was first observed in lung adenocarcinoma in which $\alpha 11\beta 1$ was identified as a tumor biomarker¹⁰⁹. Specifically, integrin $\alpha 11\beta 1$ is found to be overexpressed in the tumor stromal tissue of lung adenocarcinoma, where it induces IGF2 expression and tumorigenicity⁴⁹. Furthermore, Integrin $\alpha 11\beta 1$ is overexpressed in the tumor stroma of head and neck squamous cell carcinoma and its expression positively correlates and co-localizes with the expression of

α SMA, a myofibroblast marker with prognostic value in head and neck squamous cell carcinoma¹⁰⁶. Additionally, the gene encoding for integrin $\alpha 11\beta 1$ (ITGA11), next to six other genes, was found to promote invasion, in a spheroid based model for the invasion of breast cancer cells¹¹⁰. Moreover, integrin $\alpha 11\beta 1$ plays a role in the paracrine signaling between fibroblasts and cancer cells within tumor stroma. A study using 3D-heterospheroids composed of mouse embryonic fibroblasts (MEFs) and A549 lung carcinoma cells showed that CXCL5 expression in tumor cells was inversely related to integrin $\alpha 11\beta 1$ in MEFs, indicating that integrin $\alpha 11\beta 1$ increases the autocrine secretion of CXCL5 by lung carcinoma cells¹¹¹. Franco-Barraza *et al.* have very recently introduced that desmoplastic traits, prognostic of neoplastic recurrence, dependent on integrin $\alpha 5\beta 1$, expressed on myofibroblasts in pancreatic cancer, are maintained by matrix-regulated integrin $\alpha \nu \beta 5$ ¹⁰⁷. In this work, the author's identified a CAF phenotype, with high expression of active integrin $\alpha 5\beta 1$. Finally they propose a novel prognostic tool, in which they use stromal localization and levels of active Smad 2/3 and integrin $\alpha 5\beta 1$ to distinguish patient-protective from patient-detrimental desmoplasia, to foretell pancreatic cancer recurrence

In addition to fibroblasts, integrins on macrophages also play a key role in tumor stroma. A recent study has implicated integrins in regulating the ability of TAMs to promote tumor progression. For example, in a melanoma model, an osteopontin-rich matrix activates TAMs through ligation of integrin $\alpha 9\beta 1$, stimulating the migration of endothelial and cancer cells via prostaglandin E2 production¹¹². Similarly, the ECM protein periostin, secreted by glioblastoma stem cells, promote TAM recruitment to tumors via activation of $\alpha \nu \beta 3$ ¹¹³.

These studies indicate that integrin $\alpha 11\beta 1$, $\alpha 5\beta 1$, $\alpha 9\beta 1$ and $\alpha \nu \beta 3$ play a crucial role in the tumor stroma by controlling the phenotype and behavior of key stromal cells. The integrins $\alpha 11\beta 1$, $\alpha 9\beta 1$ and $\alpha \nu \beta 3$ show potential as therapeutic targets. In the context of drug delivery integrin $\alpha 11\beta 1$ presents as a target with high potential, since the expression of this integrin is restricted to tumor stroma or other fibrotic disease but is generally not expressed in other tissues of the adult body. The design of novel integrin targeting ligands is therefore a highly interesting approach for drug delivery to CAFs.

5.3.4 Metastasis

Metastasis, which is defined as the spreading of cells from the primary tumor site to other organs is responsible for more than 90% of cancer-related lethality

¹¹⁴. Integrins, which are regulators of cell attachment to the ECM as well as cell migration and therefore have a crucial part in the regulation of metastasis ¹¹⁵.

Integrin $\alpha 2$ is widely expressed which makes it difficult to determine its function during tumorigenesis. High expression of integrin $\alpha 2$ in breast and prostate cancer correlates with a favorable prognosis ¹¹⁶. Additionally, MMTV-neu mice lacking integrin $\alpha 2$ expression had increased tumor cell intravasation indicating that integrin $\alpha 2$ has metastasis suppressing properties ¹¹⁶. Additionally, integrin $\alpha 2\beta 1$ expression causes a decrease in lymph node metastasis in human papilloma virus-induced squamous cell carcinoma in mice ¹¹⁷. In contradiction, elevated levels of integrin $\alpha 2\beta 1$ accelerated experimental metastasis in melanoma, gastric and colon cancer ¹¹⁸⁻¹²⁰. Moreover, integrin $\alpha 2\beta 1$ rich xenograft tumors, in mice have shown to promote metastasis to the bone ¹²¹.

The role of integrin $\alpha 2$ in tumorigenesis and metastasis is not yet clarified and appears to be dependent on expression levels and the tumor type ⁶⁴. Zeltz and Gullberg ⁶⁴ hypothesized that high expression of integrin $\alpha 2$ in well-differentiated tumors might prevent metastasis, while tumors at other stages with low integrin $\alpha 2$ expression might support dedifferentiation and metastasis by directing metastasizing cells to collagen rich tissues, such as bone. In summary, integrin $\alpha 2\beta 1$ could be further exploited as a biomarker. Since studies in integrin $\alpha 2$ knockout mice show no significant side effects integrin $\alpha 2\beta 1$ additionally presents a potential therapeutic target to attenuate metastasis, but this strategy seems only applicable for not-well-differentiated tumors with low collagen expression.

In a clinical study including lung cancer patients with brain metastases (BM), high expression of integrin $\alpha v\beta 5$ on vascular structures and tumor stroma (in BM) was found to be associated with high hypoxia inducible factor 1 α (HIF1 α) indices, while $\alpha v\beta 3$ was expressed on vascular structures and tumor cells (in BM) and was correlated with low Ki-67 indices ¹²². Although it would be of high interest to see what effects new therapeutic agents against $\alpha v\beta 5$ might have on the expression of HIF1 α in BM, it could be argued that αv integrins are not a feasible therapeutic target, because blocking of these integrins could be associated with severe side effects, due to their wide expression in the human body. Still the expression of αv integrins are of pathological and clinical relevance in lung cancer patients with brain metastasis and it might be interesting to explore them as biomarkers.

In colorectal cancer, CD98, integrin $\beta 1$, $\beta 3$ and FAK was overexpressed and correlated with cancer progression and metastasis in the liver ¹²³. Direct contact between the tumor stroma and tumor cells is required for these markers to be over-expressed in metastasis of the liver ¹²³.

Integrins seem to play a crucial role in the regulation of metastasis within certain tumors. Especially integrin $\alpha 2 \beta 1$ has, dependent on tumor stage and type, potential as a therapeutic target in metastasis, while integrin $\alpha \nu \beta 3$, $\alpha \nu \beta 5$, $\beta 1$ and $\beta 3$ should be validated for their use as biomarkers. Unfortunately, none of the integrins discussed show a very specific expression in pathological tissues, but are widely expressed, which makes these integrins poor targets for drug targeting.

Figure 2. Schematic representation of the 24 different integrin receptor pairs, including their ligand specificity and cellular expression in fibrosis, tumor stroma and wound healing. The integrins cellular expression has been subdivided into non-pathogenic cell, named “normal”, and pathogenic cells, named “disease”.

Table 2. Integrins and their function in wound healing, tumor stroma, metastasis, kidney, liver and lung fibrosis.

Integrin	Expressional Modification	Function	Cell / Tissue Type/Disease	Ref.
Wound Healing				
$\beta 3$	Genetic knockdown	Accelerated re-epithelialization, enhanced TGF β signaling, dermal fibroblast infiltration	Fibroblasts, Epithelial cells	39
$\beta 1$	Fibroblast specific knockout	Delayed cutaneous wound closure and reduced granulation tissue formation, including reduced production of new ECM and reduced α SMA expression	Fibroblasts	52
$\alpha 3\beta 1$	Epidermal knockout	Impaired angiogenesis	Keratinocytes, Endothelial cells	57
$\alpha 5\beta 1$	Overexpression	Interaction of overexpressed integrin $\alpha 5\beta 1$ leads to T Cell driven keratinocyte proliferation	Keratinocytes	59
$\alpha 5\beta 1$	None	Granulation tissue fibroblasts have a reduced ability to bind fibronectin via $\alpha 5\beta 1$, increasing their migration ability	Fibroblasts	60
$\alpha 9$	None	Regulates migration and adhesion of dermal fibroblasts during granulation tissue formation in excisional wounds.	Dermal Fibroblasts	63
$\alpha M\beta 2$	Genetic knockdown	Delayed wound re-epithelialization	Neutrophils, monocytes	67
$\alpha v\beta 6$	Overexpression	Spontaneous wound development with progressive fibrosis	Epithelial cells	124
$\alpha v\beta 6$	Genetic knockdown	Delayed wound healing	Epithelial cells	125
$\alpha v\beta 5$	None	Expressed in deep human and porcine wound during early re-epithelialization	Epidermis	126
$\alpha v\beta 5$	None	Induced in keratinocytes during late mucosal and dermal wound healing	Keratinocytes	127

Integrin	Expressional Modification	Function	Cell / Tissue Type/Disease	Ref.
$\alpha 2\beta 1$	Antibody blocking	Induces MMP-1 expression and collagen matrix denaturation in wounds	Keratinocytes	128
$\alpha 5\beta 1$	Overexpression	Affects fibronectin integration and restricts keratinocyte Migration	Keratinocytes	129,130
$\alpha 11\beta 1$	Genetic knockdown	Reduced granulation tissue formation and impaired wound contraction	Fibroblasts	131
$\alpha 3\beta 1$	None	Determines the migrational directionality of keratinocytes	Keratinocytes	132
$\beta 1$	$\beta 1$ deficient keratinocytes	Impaired migration and wound healing. $\beta 1$ deficiency is accompanied by $\alpha 6\beta 4$ downregulation	Keratinocytes	133
$\alpha 3\beta 1$	Genetic knockdown	Inhibits directional migration and re-epithelialization	Keratinocytes	134
Kidney Fibrosis				
$\beta 6$	Genetic knockdown	Prevents tubulointerstitial fibrosis	Kidney	68
$\alpha 3$	Genetic knockdown	Reduced neovascularization; delayed kidney fibrosis and neovascularization	Kidney	71,72,135
$\alpha 2\beta 1$	Genetic knockdown	Knockdown delays the maturation of the glomerular basement membrane and kidney fibrosis in Alport mice	Kidney	72
$\alpha 11\beta 1$	None	Crucial for the regulation of the myofibroblast phenotype	Myofibroblasts in UUO kidneys in mice and human fibrotic kidneys	73
$\beta 6$	Genetic knockdown	Knockdown inhibits renal fibrosis in Alport mice	Kidney	74
$\alpha v\beta 1$	None	blockade of $\alpha v\beta 1$ prevents the activation of latent TGF $\beta 1$ through direct binding by fibroblasts	Fibroblasts in the Kidney	75

Integrin	Expressional Modification	Function	Cell / Tissue Type/Disease	Ref.
$\alpha 8$	Genetic knockdown	<i>De novo</i> expression in interstitial fibroblasts and tubular endothelial cells in tubulointerstitium fibrosis. Knockdown did not inhibit tubulointerstitium fibrosis, but increased tubulointerstitium damage compared to wild type mice	Fibroblasts and endothelial cells in tubulointerstitium fibrosis	76
$\alpha 3$	None	Mediates kidney fibrosis via integrin-linked kinase through mediated loss of E-cadherin	Kidney fibrosis	77
Liver Fibrosis				
$\beta 6$	Genetic knockdown	Prevents acute biliary fibrosis	Liver	68
$\alpha 3, \alpha 6$	None	Expression of these integrins indicate a switch of hepatocytes into bile duct epithelial cells	Hepatocytes in cholestasis	78
$\beta 1, \alpha 1, \alpha 5, \alpha 6$	None	Integrin expression positively correlates with the stage of fibrosis	Liver	79
$\beta 1$	None	Integrin expression level positively correlates with the stage of fibrosis.	Chronic hepatitis C & B, PBC, PSC	80
$\beta 1$	None	Overexpressed on T lymphocytes	Alcoholic liver disease	81
$\alpha L, \alpha M, \alpha X, \alpha 4$	None	Expression levels on peripheral blood leukocytes positively correlated with liver failure	Leukocytes in liver cirrhosis	83
$\alpha L \beta 2, \alpha 4 \beta 1$	None	Integrin expression correlates with infiltrating lymphocytes	Lymphocytes in PBC	84
$\alpha v \beta 6$	None	Upregulated	Biliary atresia	85
$\beta 2$	None	Induced expression of integrin $\beta 2$ on neutrophils increases their migration and Kupffer cell release of chemotactic cytokines and growth factors	Chronic alcohol intoxication of the liver	87

Integrin	Expressional Modification	Function	Cell / Tissue Type/Disease	Ref.
α L	None	Indirectly promotes fibrosis	Lymphocytes in PBC	88
β 6	Genetic knockdown	Knockdown inhibits neutrophil infiltration and plasma transaminase activity as well as hepatic necrosis		89
β 6	Genetic knockdown	Knockdown inhibits neutrophil infiltration and plasma transaminase activity as well as hepatic necrosis	Acute cholestatic hepatitis	90
β 1	None	Responsible for the recruitment of CD16(+) monocytes into the liver	Chronic liver inflammation and fibrogenesis	91
α 5 β 1	None	Increases collagen production via integrin α 5 β 1/ECM induced changes in the cytoskeletal organization and activation of Src kinases and ERK/JNK	Hepatic stellate cells in liver fibrosis	92
α v	Genetic knockdown	Protected mice from CCL4-induced liver fibrosis and was also protective in pulmonary and renal fibrosis	Myofibroblasts	94
α 11 β 1	Genetic knockdown	Major regulator in the activation of HSCs into myofibroblasts	Myofibroblasts / Liver Fibrosis in mice	73
Lung Fibrosis				
α v β 6	Genetic knockdown	Regulates pulmonary fibrosis and inflammation by activating TGF β	Epithelial cells of lung sclerosis and pulmonary fibrosis	31
α v β 8	Genetic knockdown	Increased pro-fibrotic differentiation of lung fibroblasts by regulating TGF β activation. Increases the expression of collagen and pro-fibrotic genes.	COPD fibroblasts	98-100
α v β 5	None	Mediates TGF β induced fibrosis.	Fibroblasts of pulmonary fibrosis	101

Integrin	Expressional Modification	Function	Cell / Tissue Type/Disease	Ref.
$\alpha 3\beta 1$	Genetic knockdown	Knockdown correlates with reduced accumulation of myofibroblasts, collagen and EMT-associated genes	Pulmonary fibrosis	103,104
$\alpha 11\beta 1$	none	Expression correlated concomitantly with the expression of various fibrotic parameters in the lungs of patients with IPF	Idiopathic pulmonary fibrosis	73
Tumor Stroma				
$\alpha 11\beta 1$	Genetic knockout	Expression positively correlates with prognosis. Induces IGF2 expression and tumorigenicity. Induces CXCL5 secretion by lung carcinoma cells.	Fibroblasts in Lung adenocarcinoma	49,109,111
$\alpha 11\beta 1$	None	Overexpressed in the tumor stroma. Expression positively correlates and co-localizes with the expression of α SMA	Head and neck squamous cell carcinoma	106
$\alpha 5\beta 1$	None	Desmoplastic traits prognostic of neoplastic recurrence of integrin $\alpha 5\beta 1$ are maintained by matrix regulated integrin $\alpha \nu \beta 5$	Cancer-associated fibroblasts in pancreatic cancer	107
$\alpha 11\beta 1$	None	Promotes invasion	Invasive breast cancer cells	110
$\alpha 9\beta 1$	None	Osteopontin-rich matrix activates TAMs through ligation of integrin $\alpha 9\beta 1$, stimulating the migration of endothelial and cancer cells via prostaglandin E2 production	Melanoma model	112
$\alpha \nu \beta 3$	None	Periostin, secreted by glioblastoma stem cells, promote TAM recruitment to tumors via integrin $\alpha \nu \beta 3$ signaling.	Glioblastoma xenografts	113
Metastasis				
$\alpha 2\beta 1$	Genetic knockdown	Expression is associated with favourable prognosis and reduced tumor cell intravasation	Breast Cancer, squamous cell carcinoma	116,117

Integrin	Expressional Modification	Function	Cell / Tissue Type/Disease	Ref.
$\alpha 2\beta 1$	None	Accelerated levels increase experimental metastasis	metastasis in melanoma, gastric and colon cancer	118-120
$\alpha v\beta 5$	None	Expressed in vascular structures and tumor stroma and associated with high hypoxia inducible factor 1 α indices	Brain metastasis of lung cancer	122
$\alpha v\beta 3$	None	Expressed on vascular structures and associated with low Ki-67 indices	Brain metastasis of lung cancer	122
$\beta 1, \beta 3$	None	Overexpressed and correlate with cancer progression and metastasis in the liver.	Liver metastasis of colorectal cancer	123

5.4 Therapies based on integrins inhibition

Various different integrins have been identified to play a role in fibrosis and their knockdown or blocking has been shown to dampen disease progression. An example of a clinically approved integrin inhibitor is the integrin $\alpha 4\beta 7$ inhibitor vedolizumab, selectively inhibiting lymphocyte trafficking, which is applied as a treatment in Crohn's disease ¹³⁶. Therefore integrin-specific inhibitors have a huge potential as anti-fibrotic therapeutics. An overview of therapies (pre-clinical and clinical) based on integrin inhibition are described in the following section and are summarized in Table 3.

5.4.1 αv -Family integrins

The majority of integrin targeting drugs tested in clinical trials inhibit αv integrins. In the context of this review, it is important to realize that αv integrins are generally expressed in the blood vessels and various other endothelial tissues and in the case of cancer, these drugs may target tumor cells, next to angiogenic vessels of the tumor microenvironment. Although integrins are abundantly present on tumor cells, in this review we focus on tumor stromal cells and we therefore only mention integrin αv inhibiting therapies, which clearly demonstrated effects on these cells.

An antibody so-called vitaxin against $\alpha\beta 3$, which was later evolved into etaracizumab was tested in phase I and II clinical trials with low toxicity. Therapeutic efficacy but no immunogenicity was observed after treatment with etaracizumab in metastatic melanoma and other solid tumors. However, further development of etaracizumab was terminated based on the results of a randomized clinical trial in which its efficacy was compared to standard chemotherapy showing no meaningful improvement¹³⁷⁻¹⁴⁰.

Another antibody against $\alpha\beta 3$ and $\alpha\beta 5$, CTNO 95, has been tested in phase I clinical trials in advanced solid tumors showing anti-tumor activity and no toxicity¹⁴¹. Hereupon, CTNO 95 together with docetaxel and prednisolone was evaluated in a multicenter phase II clinical trial for safety and efficacy in patients with castration-resistant prostate cancer in which CTNO 95 caused a shorter progression free survival without showing additional toxicity compared to placebo treatment¹⁴². Later CNTO 95 was tested in combination with bevacizumab in a phase I biomarker study in patients with advanced solid tumors, could be administered safely and resulted in changes of the plasma levels of soluble endoglin, soluble E-cadherin, and soluble E-selectin as well as PIGF and VEGF-D, all proteins which interact with the ECM¹⁴³. The selective $\alpha\beta 3$ and $\alpha\beta 5$ blocker cilengitide, based on the cyclic RGD peptide was successfully tested in phase I and II clinical trials for lung cancer, prostate cancer and glioblastoma but failed to enhance the survival benefit in patients when cilengitide was given in addition to the standard of care therapy¹⁴⁴⁻¹⁴⁷. Using a small molecule inhibitor for integrin $\alpha\beta 1$, Reed *et al.*¹⁴⁸ found that $\alpha\beta 1$ directly binds to the latency-associated peptide of TGF $\beta 1$, thereby mediating TGF β activation. Administration of this small molecule inhibitor showed therapeutic efficacy by attenuating bleomycin-induced pulmonary fibrosis and carbon tetrachloride-induced liver fibrosis but has not been evaluated in clinical trials¹⁴⁸.

Antibody mediated blocking of the TGF β activating integrin $\alpha\beta 6$ has shown therapeutic activity in a wide range of pre-clinical fibrosis models. These models include models for lung fibrosis^{149,150}, liver fibrosis^{151,152} and renal fibrosis^{74,153}. A humanized monoclonal antibody, STX-100 (BG00011), against $\alpha\beta 6$ is currently being tested in phase 2 clinical trials in patients with idiopathic pulmonary fibrosis¹⁵⁴. Genetic knockdown of $\beta 6$ and functional antibody blocking of $\alpha\beta 6$ in renal fibrosis attenuates the accumulation of activated fibroblasts and interstitial collagen matrix deposition⁷⁴. Treatment of scleroderma fibroblasts with antibodies against integrin $\alpha\beta 3$ and $\alpha\beta 5$ reduced

the expression of procollagen type I^{33,34,40,155,156}. Antibody mediated blocking of the integrins $\alpha\beta 3$ and $\alpha\beta 5$ inhibits myofibroblast differentiation in oral and dermal fibroblasts *in vitro*, while the inhibition of differentiation of kidney fibroblasts was only achieved with antibody blocking of $\alpha\beta 3$ ¹⁵⁷. A monoclonal antibody against $\alpha\beta 3$ blocked fibroblast growth factor (FGF), tumor necrosis factor-alpha and human melanoma fragments induced angiogenesis in human wound granulation tissue¹⁵⁸. A small molecule inhibitor of $\alpha\beta 6$ (EMD527040) inhibited bile duct proliferation and peribiliary collagen deposition, decreased the expression of pro-fibrotic and induced fibrolytic genes¹⁵⁹. In Mdr2(Abcb4)(-/-) mice with spontaneous biliary liver fibrosis, a single dose of a selective $\alpha\beta 6$ inhibitor significantly induced profibrolytic MMP-8 & -9, and showed downregulation of the fibrosis markers procollagen $\alpha 1$, TGF $\beta 2$ and MMP-2⁸⁰.

The integrins $\alpha\beta 3$ and $\alpha\beta 6$ targeting peptide ACDCRGDCFC has been conjugated to the pro-apoptotic antimicrobial synthetic peptide (KLAKLAK)₂ and has shown selective toxicity to angiogenic endothelial cells, by disrupting their mitochondrial membranes, and showed anti-cancer activity in mice¹⁶⁰.

Hepatic stellate cells, precursors of liver myofibroblasts, are one of the major sources of ECM production in liver fibrosis, which makes them a target for anti-fibrotic therapeutics. Zhou *et al.* found that inhibition of integrin $\alpha\beta 3$ with neutralizing antibodies, echistatin or small inhibitory RNA to silence the α subunit expression, decreased proliferation of hepatic stellate cells¹⁶¹. More recently, the $\alpha\beta 3$ inhibitor Cilengitide was used to treat liver fibrosis in rat, induced by bile duct ligation (BDL) or thioacetamide (TAA) injections, and resulted in a significant decrease of liver fibrosis and collagen deposition, but increased experimental liver fibrosis (~30%)¹⁶². By blocking both ICAM-1 and integrin α on hepatic stellate cells, phagocytosis of fibrosis promoting lymphocytes, a process mediated through members of the Rho family (Cdc42 or Rac-1) and leading to the activation of hepatic stellate cells was completely prevented¹⁶³.

Genetic knockdown and blocking of integrin $\alpha\beta 6$ with antibodies prevented radiation-induced pulmonary fibrosis¹⁵⁰ and hepatic fibrosis induced by biliary obstruction in mice¹⁵¹. The small molecule inhibitor C8 binding to $\alpha\beta 1$ in picomolar concentrations, significantly inhibited liver and lung fibrosis in mice, reducing collagen deposition by approximately 50%¹⁴⁸. In a renal unilateral obstruction model, administration of C8 inhibited collagen deposition and effectively attenuated renal failure⁷⁵. Another small molecule inhibitor blocking

α v, CWHM 12, attenuated CCl₄-induced liver fibrosis, bleomycin-induced lung fibrosis as well as cerulein-induced pancreatic fibrosis in mice ^{94,164}. Although, most therapeutic approaches inhibiting integrins are directed against α v there are only a handful of compounds which have made it to clinical trials and those have failed to show improved therapeutic efficacy, which might be related to off-target binding of these inhibitor due to the wide expression of integrin subunit α v.

5.4.2 α 5-Family integrins

An additional target for anti-cancer therapy is integrin α 5 β 1, known to be expressed on CAFs, angiogenic vessels and tumor cells. A humanized monoclonal antibody, specifically binding to α 5 β 1, volociximab, showed absence of severe toxicities in patients with solid tumors and resulted in one minor response and disease stabilization in another case in a phase I clinical trial ¹⁶⁵. A phase II study with volociximab was performed in patients with relapsed malignant melanoma showing insufficient effects to proceed to stage 2 of the study ¹⁶⁶. The subsequent phase II study evaluating volociximab in refractory metastatic clear cell renal cancer resulted in stable disease in 87 % of the patients ¹⁶⁷. This study is continued in a follow-up in which higher dose levels are being evaluated patients ¹⁶⁷.

In metastatic pancreatic cancer, volociximab was studied in combination with gemcitabine in which 5% had confirmed partial response and 50% of the patients showed a stable disease ¹⁶⁸. In a phase II study in platinum resistant advanced epithelial ovarian or primary peritoneal cancer, volociximab showed insufficient clinical activity ¹⁶⁹. Another study in recurrent ovarian or primary peritoneal cancer, volociximab was used in combination with pegylated liposomal doxorubicin, showing no statistically significant difference when compared to pegylated doxorubicin alone ¹⁷⁰. In a phase I dose escalation study of volociximab in combination with carboplatin and paclitaxel in patients with advanced non-small cell lung carcinoma showed promising clinical efficacy, but the development of this therapy has not been developed further ¹⁷¹. Another integrin α 5 β 1 monoclonal antibody developed by Pfizer, PF- 4605412 has also been evaluated in phase I clinical trials and has been discontinued due to the acute infusion-related reactions ¹⁷².

An integrin α 5 β 1 binding RGD peptide inhibited the progression of CCl₄-induced liver fibrosis and collagen deposition in the liver ¹⁷³. Additionally, RGD inhibited the expression of collagen 1 and tissue inhibitor of MMP-1 and

increased MMP-1 expression of human hepatic stellate cell derived cells *in vitro* ¹⁷³.

Another $\alpha 5\beta 1$ targeting peptide derived from the synergy region of fibronectin, binding to $\alpha 5\beta 1$ and $\alpha v\beta 3$, ATN-161 (Ac-PHSCN-NH₂) caused a dose-dependent decrease in tumor volume and inhibited metastasis in a metastatic mouse breast cancer model ¹⁷⁴. In a phase I clinical trial in patients with solid tumors, ATN-161 caused no dose limiting toxicities ¹⁷⁵. Unfortunately, no objective responses to ATN-161 treatment was found, but prolonged stable disease was observed in patients with renal cancer ¹⁷⁵.

5.4.3 $\alpha 2$ -Family integrins

A small molecule inhibitor of integrin $\alpha 2\beta 1$ (E7820) was investigated in phase I clinical trials for the treatment of metastatic colon cancer and is currently tested in phase II clinical trials in combination with certuximab ^{176,177}

5.4.4 $\beta 1$ -Family integrins

Since integrin $\beta 1$ is a partner receptor for many α receptor units, it is present ubiquitously. In bevacizumab-resistant glioblastomas (BRG) integrin $\beta 1$ mediates interactions between the tumor and its microenvironment ¹⁷⁸. These interactions include tumor cell binding to ECM-ligands like fibronectin, collaten IV and laminin, and VEGF-dependent as well as independent vascularization ¹⁷⁸. Treatment of BRG mouse xenografts with the integrin $\beta 1$ specific antibody, OS2966, allowed a reduction in the dose of bevacizumab and delivery of OS2966 over a period of 28 days showed increased apoptosis of tumor cells and a decrease in tumor cell invasiveness and mesenchymal morphology of tumor cells ¹⁷⁸.

A 41 amino acid peptide, purified from the venom of the *Viperia lebetina obtusa* viper is an effective inhibitor of integrin $\alpha 1\beta 1$ and inhibited angiogenesis and tumor growth *in vivo* in the chicken chorioallantoic membrane assay and the Lewis lung syngenic mouse model ¹⁷⁹.

5.4.5 αL , αM and $\beta 2$ -Family integrins

αL and αM -family integrins antibody mediated blocking of integrin $\alpha L\beta 2$ in mice that underwent bile duct ligation decreased the activity of alanine aminotransferase and aspartate aminotransferase levels in serum. Additionally the adhesion of leukocytes in bile duct ligation induced post-sinuoidal venules

was reduced ¹⁸⁰. Integrin α M and CD44 blockage with antibody reduced the localization of monocytes to hepatic foci ¹⁸¹. Additionally, treatment of rats with the neutropenic monoclonal antibody 1F123 against integrin β 2, capable of forming heterodimer with integrin α M, α L, α X and α D, attenuated alcohol initiated hepatic injury ⁸⁷.

Table 3. Therapeutics for integrin inhibition in development.

Integrin Target	Compound Name	Stage (year)	Cellular Target	Disease Target	Ref.
αv-Family Integrins					
α v β 3	Vitaxin	Phase I (2000)	Endothelial Cells	Breat, Lung and Colon Cancer	137
α v β 3	Etaracizumab	Phase I (2005, 2008)	Endothelial Cells	Several solid tumors	138,139
α v β 3	Etaracizumab	Phase II (2010)	Endothelial Cells	Metastatic melanoma	140
α v	CTNO 95	Phase I (2007)	Endothelial and Tumor Cells	Several solid tumors	141
α v	CTNO 95	Phase II (2013)	Endothelial and Tumor Cells	Castration-resistant prostate cancer	142
α v	CTNO 95	Phase I (2015)	Endothelial and Tumor Cells	Several solid tumors	143
α v β 3, α v β 5	Cilengitide	Phase II (2006)	Endothelial and Tumor Cells	Prostate Cancer	144
α v β 3, α v β 5, α v β 1	Cilengitide	Phase I (2007) Phase II (2008) Phase III (2014)	Endothelial and Tumor Cells	Malignant Glioma, Glioblastoma	145,146,182
α v β 1	C8	Pre-clinical	Cancer-associated fibroblasts	Liver fibrosis, Lung fibrosis, Kidney fibrosis	75,148

Integrin Target	Compound Name	Stage (year)	Cellular Target	Disease Target	Ref.
$\alpha v \beta 1$	c8	Pre-clinical	Activated fibroblasts	Bleomycin-induced pulmonary fibrosis, Carbon tetrachloride-induced liver fibrosis	148
αv	CWHM 12	Pre-clinical		CCL4-induced liver fibrosis, bleomycin-induced lung fibrosis, cerulein-induced pancreatic fibrosis	94,164
$\alpha v \beta 6$	Anti- $\alpha v \beta 6$ -mAb	Pre-clinical	Acinar cells, Pancreatic Stellate Cells	Lung Fibrosis, Biliary Fibrosis, Liver Fibrosis, Renal Fibrosis, Kidney Fibrosis	74,150-153
$\alpha v \beta 5$	PIF6	Pre-clinical	Kidney fibroblasts, Oral fibroblasts, Dermal fibroblasts	/	157
$\alpha v \beta 3$	LM609	Pre-clinical	Oral fibroblasts, Dermal fibroblasts	/	157
$\alpha v \beta 6$	STX-100	Phase II (2017)	/	Pulmonary Fibrosis	154
$\alpha v \beta 3$	Anti- $\alpha v \beta 3$ -mAb	Pre-clinical	Endothelial Cells	Human Wound Tissue	158
$\alpha v \beta 6$	EMD527040	Pre-clinical	Bile duct epithelial cells, Mdr2(Abcb4)(-/-) mice with spontaneous biliary fibrosis	Liver Fibrosis	80,159

Integrin Target	Compound Name	Stage (year)	Cellular Target	Disease Target	Ref.
$\alpha v\beta 3$, $\alpha v\beta 6$	ACDCRGDC FC-(KLAKLAK) ₂	Pre-clinical	Endothelial cells	/	160
$\alpha v\beta 3$	Echistatin, αv RNAi, anti $\beta 3$	Pre-clinical	Hepatic stellate cells	Liver Fibrosis	161
$\alpha v\beta 3$, $\alpha v\beta 5$	Cilengitide	Pre-clinical	Hepatic stellate cells	Experimental liver Fibrosis	162
αv	Anti-Integrin alpha V	Pre-clinical	Hepatic Stellate Cells	Liver Fibrosis	163
$\alpha 5$-Family Integrins					
$\alpha 5\beta 1$	Volociximab	Phase II (discontinued, 2006 - 2011)	Endothelial Cells	Ovarian cancer, peritoneal cancer, pancreatic cancer, renal cancer	166-170
$\alpha 5\beta 1$	Volociximab	Phase I (discontinued, 2008, 2013)	Endothelial Cells	Advanced Solid Malignancies, Non-small-cell lung cancer	165,171
$\alpha 5\beta 1$	RGD	Pre-clinical	Hepatic stellate cells	Liver cirrhosis	173
$\alpha 5\beta 1$	PF-4605412 (Mab)	Phase I (discontinued, 2013)	Endothelial Cells	Solid Malignancies	172
$\alpha v\beta 3$, $\alpha 5\beta 1$	ATN-161	Pre-clinical	Endothelial Cells	Breast cancer	174
$\alpha v\beta 3$, $\alpha 5\beta 1$	ATN-161	Phase I (2006)	Endothelial Cells	Solid tumors	175
$\alpha 2$-Family Integrins					
$\alpha 2\beta 1$	E7820	Phase I (2011)	Endothelial Cells	Advanced solid tumors	176
$\beta 1$	OS2966	Pre-clinical	Endothelial Cells	Glioblastoma	178
$\alpha 1\beta 1$	Obtustatin	Pre-clinical	Endothelial Cells	Lung cancer	179

αL, αM and β2-Family Integrins					
α L β 2	anti-LFA-1 antibody	Pre-clinical	Leukocytes	Liver Fibrosis	180
α M	Anti-CD11b antibody	Pre-clinical	Monocytes	Liver Infection	181
β 2	1F12	Pre-clinical	Neutrophils in the liver	Experimental alcoholic hepatitis	87

5.5 Drug targeting strategies based on integrin ligands

Extensive work has been done in the design of new ligands for integrin receptor targeting that can be utilized for integrin facilitated drug delivery or imaging. A large portion of these ligands are targeting RGDs and have been extensively reviewed in a recent article by Arosio *et al.* ¹⁸³. Therefore, RGD based targeting approaches have been excluded from this review, unless it has been shown that they have facilitated specific targeting to components related to fibrosis, wound healing or the tumor stroma.

Ruoslahti *et al.* have developed two peptides binding to the α v binding motifs Arg-Gly-Asp (RGD) and Asn-Gly-Arg (NGR), and coupled these to the anti-cancer drug doxorubicin. Targeting the tumor vasculature of human breast cancer xenografts in mice, resulted in enhanced efficacy of doxorubicin ¹⁸⁴. Targeting doxorubicin to endothelial cells by linking a bicyclic CDCRGDCFC (RGD-4C) peptide selectively binding to integrin α v β 3 and α v β 5 and a D-Ala-Phe-Lys tripeptide selectively binding to tumor-associated protease plasmin to the drug, resulted in plasmin-dependent cytotoxicity of endothelial cells ¹⁸⁵. The cyclic RGD compound containing the conformational constrained homoSer-Pro dipeptide unit with a fluorescent probe for α v β 3-imaging has shown to possess the ability to bind endothelial cells *in vitro* ¹⁸⁶. A new α v β 3-specific tumor vessel binding cyclic peptide containing the iso-DGR motif was conjugated to albumin and coupled to tumor necrosis factor- α (TNF- α) carrying gold nanoparticles ¹⁸⁷. This nanodrugs showed inhibited tumor growth in WEHI fibrosarcoma bearing mice ¹⁸⁷.

The integrin α 5 β 1 antagonistic peptide ATN-161 in combination with the chemotherapeutic agent 5-fluorouracil significantly reduced liver metastasis, tumor microvessels as well as increased tumor cell apoptosis and decreased tumor cell proliferation, resulting in an increased overall survival in mice ¹⁸⁸.

Later, ATN-161 was investigated in phase II trials in cancer¹⁸⁹. The PHSCNK peptide targeting $\alpha 5 \beta 1$ was applied to modify Dox-loaded liposomes to enable targeting of the tumor vasculature and showed enhanced cytotoxicity to endothelial and breast cancer cells, which was attributed to integrin mediated endocytosis¹⁹⁰. Mardilovich *et al.* have designed a integrin $\alpha 5 \beta 1$ specific peptide PR_b, mimicking the cell binding site of native fibronectin¹⁹¹. Later this peptide has been applied to target siRNA and encapsulated in polyerosomes, lipoplexes, polyplexes and stealth liposomes to integrin $\alpha 5 \beta 1$ ¹⁹²⁻¹⁹⁴.

Figure 3. Existing integrin drug targeting ligands, applied for the modification of drug molecules, drug carriers and CAR-T cells (CAR-T), in the context of wound healing, fibrosis or tumor stroma and their respective integrin targets. Dashed lines show potential integrin targets for existing ligands that have not yet been used for targeting the respective cell line. In addition to already targeted integrins, integrins that have not yet been targeted but play a role in the pathology of wound healing, fibrosis or tumor stroma are presented.

Peptidomimetic integrin ligands selectively binding to either $\alpha 5 \beta 1$ or $\alpha v \beta 3$ in fibroblasts functionalized with 1-((1,3-dicarboxypropyl)-4,7-(carboxymethyl)-

1,4,7-triazacyclononane (NODAGA) successfully targeted $\alpha 5\beta 1$ or $\alpha v\beta 3$ expressing tumors in mice. Additionally, both peptidomimetics inhibited tumor growth in syngeneic subcutaneous WEHI-164 fibrosarcomas in mice by inhibiting $\alpha 5\beta 1$ or $\alpha v\beta 3$, respectively^{195,196}.

Modification of cisplatin with platinum (IV) complexes conjugated to the $\alpha v\beta 3/\alpha v\beta 5$ -specific peptide motifs RGD, (CRGDC)_c, (RGDfK)_c or NGR to target tumor endothelial cells, showed anti-proliferative effects on human endothelial cells *in vitro*¹⁹⁷. Pan *et al.*, have modified PEGylated perfluorooctylbromide (PFOB) nanoparticles (NPs) carrying the anti-angiogenic fumagillin with a quinolonic $\alpha v\beta 3$ -integrin ligand conjugated to the PEG chain of these NPs. This drug delivery system decreased angiogenesis in a Matrigel plug assay for angiogenesis in mice¹⁹⁸. Fu *et al.*, have modified chimeric antigen receptor (CAR) T cell with echistatin, having a high binding affinity to integrin $\alpha v\beta 3$. The echistatin expressing T cells were capable to efficiently lyse HUVECS, resulting in the extensive bleeding of the tumor tissue *in vivo* without damaging blood vessels in normal tissues and facilitated specific penetration of liposomes into the tumor site¹⁹⁹.

5.6 Conclusions and future directions

Despite a lot of work being done to gain insight in the biological role of integrin subtypes in wound healing, fibrosis and tumor stroma, there is still a need to better understand their significance and implications. Most studies have been carried out by the means of integrin subunit knockout in mice or using integrin-specific inhibitory antibodies. A few studies have revealed integrins with a role in fibrosis, which resulted in some potential anti-fibrotic therapeutics. However, most of them failed in early or late clinical trials. It is worth mentioning that integrin αv and $\beta 1$ are the most studied integrin receptors because they are the partner receptors of many heterodimeric receptors. RGD and its derivative peptide sequences binding to αv and $\beta 1$ units are the most studied ligands against integrins with therapeutic potential. However, most of them are not specific to one but various integrin receptors.

Furthermore, it is very important to realize that integrin αv and $\beta 1$ are widely expressed within the human body and are not specifically expressed. Some of the cells types which express integrin αv and $\beta 1$ include e.g. endothelial cells, tumor cells. Therefore, molecules targeting αv or $\beta 1$ intergrins are prone to exert off target effects. This could also explain why inhibitors such as Cilengitide,

which have made it to phase III clinical trials have finally been terminated. Especially in fibrosis related diseases, it could be an interesting approach to develop molecules targeting integrins with a more disease-specific expression which are not involved in angiogenesis but for example expressed on myofibroblasts or tumor associated macrophages, two of the main drivers of disease progression in fibrosis, tumor stroma and wound healing. Therefore, studies exploiting the expression of a specific integrin subunit and its cellular localization within pathological tissues and other healthy organs are crucial to determine novel integrin-based therapeutic targets. Moreover, the design of integrin subunit specific drugs will decrease the risk of off-target effects due to a high specificity of these molecules. An additional major aspect for the identification of integrin subunits as therapeutic targets is to have a clear understanding of integrin subunit – growth factor receptor interactions.

Furthermore, with regard to drug targeting approaches, mainly targets of RGD-based targeting peptides binding to integrin $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha v \beta 6$ and $\alpha 5 \beta 1$ have been applied. Also in this context targeting of non-disease-specific integrins and integrin subunits can lead to off target effects and could result in severe toxicities, depending on the payload of the delivery system, even if the targeting molecule itself does not exert toxic effects.

Another important point to consider with regard to RGD-based targeting approaches is a finding by Kunjachan *et al.*²⁰⁰ which showed that passive targeting of 10 nm-size polymeric nanocarriers resulted in enhanced intratumoral nanoparticle accumulation and retention when compared with RGD-peptide modified polymeric nanoparticles. These observations might be different for targeting systems with a larger size but it should be taken into consideration that targeting approaches directed towards targets with a more specific expression within a certain disease might increase site-specific accumulation and retention and additionally reduce side effects.

In conclusion, studies which have revealed the biological function of various integrins within fibrosis related disease, although not completely understood, imply various novel therapeutic and drug targeting targets. One of the major challenges remaining is to use the knowledge obtained on integrin biology to develop highly integrin subunit specific targeting molecules, which can be applied as therapeutics by themselves or utilized as targeting ligands in disease-specific drug delivery systems.

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Chapter 6 - Integrin $\alpha 11$ regulates differentiation of pancreatic stellate cells in pancreatic cancer

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Abstract:

Pancreatic ductal adenocarcinoma (PDAC) is the deadliest tumor due to its highly abundant tumor stroma. One of the main components of the tumor stroma are pancreatic stellate cells (PSCs), precursor cells of cancer-associated fibroblasts (CAFs), which support PDAC progression by enhancing tumor cell growth, invasion and metastasis. Integrins are extracellular matrix receptors playing a crucial role in cell adhesion, migration and function of several growth factors. In the presented study, we aimed to investigate the expression of integrin $\alpha 11$ (ITGA11) in human PDAC and study the role of ITGA11 in PSCs activation and function.

In this study, we show the immunohistological localization of ITGA11 for the first time in human PDAC specimens. We found that ITGA11 is highly expressed on stromal myofibroblasts of PDAC patients, as confirmed by co-localization with the myofibroblast marker α -smooth muscle actin (α -SMA). In contrast, there was no expression in healthy pancreas and other human organs. Activation of primary human PSCs (hPSCs) with TGF- β or conditioned medium from Panc-1 (Panc-1 CM) resulted in the significant upregulation of ITGA11 and various markers which were previously shown to be upregulated in activated hPSCs and CAFs (e.g. α -SMA, Collagen-I). Stable ITGA11 knockdown (shITGA11), significantly inhibited TGF- β and Panc-1 CM mediated induction of major hPSC activation markers (such as α -SMA, Collagen-I) on gene and protein level. Strikingly, various cytokines, growth factors, cell adhesion molecules and ECM components which were induced with TGF- β or Panc-1 CM in hPSCs, were reduced in shITGA11 hPSCs. These findings demonstrate that these cytokines and growth factors (e.g. MIF, TGF- $\beta 3$, IGFBP-1, 3, CXCL-1, PDGF-BB) are regulated by ITGA11 upon TGF- β or Panc-1 CM activation.

Additionally, shITGA11 hPSCs had lowered migration and contractility. To study the effect of ITGA11 on the paracrine activity of hPSCs, we applied conditioned medium from hPSCs on Panc-1 tumor cells. We found that the conditioned medium obtained from TGF- β -activated hPSCs induced the migration and invasion ability of Panc-1. These inductions were highly reduced when the conditioned medium from shITGA11 hPSCs was used, demonstrating that ITGA11 knockdown can inhibit pro-tumoral paracrine signalling between hPSC and Panc-1 tumor cells.

In conclusion, this study presents ITGA11 as an interesting therapeutic target which is highly and specifically expressed in the pancreatic tumor stroma and plays a key role in the regulation of activation and paracrine function of hPSCs.

6.1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most devastating disease in our society with a 5-year survival rate of only 8% ¹. Due to the advanced stage of the disease, a surgical resection of the tumor is not feasible in most of the patients with PDAC. Therefore, radiotherapy or cytotoxic chemotherapy are the golden standard treatment, but these treatments are not effective for PDAC which results in a survival benefit of only a few months ^{2,3}. One of the biological features that contributes to the high drug resistance, progression, invasion and metastasis of PDAC is the abundant desmoplastic reaction, also known as the tumor stroma ⁴. The pancreatic tumor stroma can take up to 90 % of the entire tumor mass and its main cellular component are cancer-associated fibroblasts (CAFs) which support PDAC progression by producing ECM proteins and the secretion of various cytokines and growth factors, which stimulate tumor growth, angiogenesis, invasion, and metastases ⁵⁻⁷.

Different studies have shown an anti-tumoral effects in pancreatic cancer by modulating its tumor stroma ^{8,9}, while on the contrary complete genetic depletion of the stroma resulted in an aggressive tumor phenotype ¹⁰. Therefore, current strategies focus on modulating the activity of tumor stroma instead of completely depleting it to gain therapeutic benefits ^{8,11-13}. In human pancreatic cancer, the main progenitors of CAFs are human pancreatic stellate cells (hPSCs) ¹⁴. In the healthy pancreas quiescent hPSCs have a cytoplasmic lipid storing capacity and are normally present in the connective tissue in low numbers, secreting only small amounts of ECM components ¹⁵. However, during malignancy quiescent hPSCs get activated, lose their cytoplasmic lipid storing capacity and secrete high amounts of ECM, which ultimately presents as a barrier for tumor drug

penetration. In addition, activated hPSCs secrete various cytokines and growth factors which stimulate tumor cells and other stromal cells inducing tumor progression, metastasis and drug resistance^{16,17}. Activated hPSCs therefore present an attractive and promising cellular target for developing therapies to modulate tumor stroma.

Cellular interaction with the extracellular environment are mostly mediated by integrins, a known family of cell surface receptors, which have been recognized for their role in myofibroblast differentiation¹⁸. In general, the integrin family consists of 24 different heterodimeric transmembrane receptors composed of different combinations of 18 different α - and 8 different β -subunits¹⁸. Since integrins build a physical connection with the inside and the outside of a cell, they are capable of conducting bidirectional signaling. Through this dynamic mechanism, integrins have the ability to control cellular cytoskeleton organization, thereby controlling crucial cellular functions such as cell adhesion, migration, proliferation, survival and differentiation^{18,19}. Integrins are also known for their interaction with growth factors, for example, αv integrins can activate latent TGF β 1^{18,20}. Additionally, integrins interact with growth factor receptors via different mechanisms to regulate their signaling pathways^{18,21}.

Integrins are therefore interesting target receptors to develop novel therapeutic strategies against tumor stroma. As collagens are the major ECM proteins in the tumor stroma, one of the major collagen binding integrin $\alpha 11\beta 1$ (ITGA11) is an important receptor in CAFs. ITGA11 has been shown to be overexpressed in the tumor stroma of head and neck squamous cell carcinoma, in which it positively correlated with the expression of CAF marker α -smooth muscle actin (α -SMA)²². Additionally, ITGA11 was recognized for its role in inducing tumor cell growth and metastatic potential of small cell lung carcinoma cells²³. In this study, we investigated the expression and cellular localization of ITGA11 in human PDAC samples. Next, we studied the ITGA11 expression in activated hPSCs *in vitro* and demonstrated its role in regulating hPSC activation, differentiation, migration and phenotypic changes using knockdown studies. Finally, we investigated the influence of ITGA11 knockdown on hPSC-mediated paracrine effect on tumor cells.

6.2 Materials and Methods

6.2.1 Staining of human PDAC sections

Paraffin embedded human PDAC sections and normal human pancreatic sections were obtained from LabPON (Hengelo, The Netherlands). Tissue sections were sliced, deparaffinized in xylene and rehydrated in serially diluted alcohol solutions, followed by demineralized water for 5 minutes respectively. Antigen retrieval was performed in Tris EDTA buffer (pH 9.0, Dako, Glostrup, Denmark) by heat induction at 80°C. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide (ThermoFisher Scientific, Landsmeer, The Netherlands) in methanol for 30 min.

After rinsing with MilliQ water the tissue sections were incubated overnight at 4°C with primary antibodies (Table 1) diluted in PBS. Control samples were incubated without primary antibodies in PBS overnight at 4°C. The slides were then incubated with the secondary and tertiary fluorescent antibody or HRP-labeled antibody (Table 1) diluted in PBS supplemented with 5% of normal human serum (ThermoFisher Scientific), respectively for 1 hour at room temperature (RT). Stainings performed with HRP-labeled antibodies were visualized using 3,3-diaminobenzidine tetrahydrochloride solution (Dako) for 5-10 min resulting in brown color and then counterstained with hematoxylin for 2 min. Subsequently, the tissue sections were dehydrated and mounted with VectaMount (Vector, Burlingame, USA). For staining with fluorescent antibodies, the slides were washed and mounted with fluoroshield™ with DAPI (Sigma Aldrich) for staining of nuclei. All stained sections have been imaged using a Hamamatsu NanoZoomer Digital slide scanner 2.0HT (Hamamatsu Photonics, Bridgewater NJ). Scoring was performed visually.

Table 1. Antibodies used for the staining of human PDAC and normal pancreas sections.

Antibody	Source	Dilution
Goat anti-Integrin $\alpha 11$	R&D Systems	1:250
Mouse anti- α -SMA	Sigma Aldrich	1:200
HRP-conjugated goat anti-rabbit IgG	DAKO	1:100
HRP-conjugated rabbit anti-goat IgG	DAKO	1:100
HRP-conjugated rabbit anti-mouse IgG	DAKO	1:100
Alexa Fluor 594 labelled donkey anti-goat	Life Technologies	1:100
Alexa Fluor 488 labelled donkey anti-mouse	Life Technologies	1:100

6.2.2 Cell lines

Primary human pancreatic stellate cells (ScienCell, Carlsbad, USA), were cultured in complete Stellate Cell Medium (supplemented with 2 % FBS, 1 % Penicillin/Streptomycin and 1 % Stellate Cell Growth Supplements (SteCGS)) (ScienCell). Panc-1 cancer cells were cultured in Dulbecco's Modified Eagles medium (DMEM) High Glucose (4.5 g/l) with L-Glutamine (GE Healthcare, Vienna, Austria) supplemented with 10 % FBS (Lonza, Verviers, Belgium), 100 µg/ml penicillin/streptomycin (Sigma Aldrich, Zwijndrecht, The Netherlands). The cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

6.2.3 Stable ITGA11 knockdown in hPSCs

To generate hPSCs with a stable knockdown of ITGA11 (shITGA11), primary hPSCs were transfected with either a scrambled shRNA control plasmid or shRNA ITGA11 plasmid using lipofectamine 2000 (Invitrogen, Carlsbad, USA) as described elsewhere ²⁴. After 24 hours of transfection with lipofectamine, transfected cells were selected and cultured with 1 µg/ml of puromycin (Invitrogen).

6.2.4 Quantitative real-time PCR and RT² profiler PCR array

To evaluate the effect of ITGA11 knockdown on TGF- β -mediated hPSCs activation, cells were seeded into a 12 well plate at a seeding density of 40.000 cells/ well. The cells were starved for 24 h and activated with 5 ng/µl TGF- β or Panc-1 conditioned medium. 24 h after activation, total RNA was isolated using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich). The amount of isolated RNA was measured by a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Rockford, USA). Next, cDNA was synthesized with iScript™ cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). 5 ng cDNA were used for each real-time PCR reaction. Real-time PCR primers (Table 2) were purchased from (Sigma Aldrich). Real-time PCR was performed using the 2x SensiMix SYBR and Fluorescein Kit (Bioline GmbH, Luckenwalde, Germany) with a BioRad CFX384 Real-Time PCR detection system (BioRad). For the RT² profiler PCR array for human extracellular matrix & adhesion molecules (Qiagen, Hilden, Germany), cDNA from three independent experiments was mixed and performed as described in the manufacturer's instructions and read with a BioRad CFX384 Real-Time PCR detection system (BioRad).

Table 2. Sequences of forward and reverse primers used during real-time PCR.

Gene	Forward Primer	Reverse Primer
RPS18	TGAGGTGGAACGTGTGATCA	CCTCTATGGGCCCCGAATCTT
ITGA11	CAGCTCGCTGGAGAGATACG	TTACAGGACGTGTTTCGCCTC
Acta-2	CCCCATCTATGAGGGCTATG	CAGTGGCCATCTCATTITCA
ITGA11	CAGCTCGCTGGAGAGATACG	TTACAGGACGTGTTTCGCCTC
PDGF β R	AGGCAAGCTGGTCAAGATCT	GCTGTTGAAGATGCTCTCCG
Desmin	GCGGGTTTCGGCTCTAAGG	AGAAACTCCTGGTTCACCGC
Collagen3 α 1	AAGAAGGCCCTGAAGCTGAT	GTGTTTCGTGCAACCATCCT
NSDHL	ATGCTATGGAGAGGACCGTG	CAGAAGGAGTCACAGGCTCA
CTGF	GTTTGGCCCAGACCCAACTA	GGCTCTGCTTCTCTAGCCTG
INSIG1	GGCAGCTTCCCAAGTATTCG	CTACCTCCTTTGGGCACTGA
ID1	TTGGGCTGGATAAAACCCCT	GCAACATCCGGCATAACTGT
MMP2	AGGAGGAGAAGGCTGTGTTC	CTCCAGTTAAAGGCGGCATC
CXCR-4	GCGGTTACCATGGAGGGGAT	CCCATGACCAGGATGACCAAT

6.2.5 Western blotting analysis

Cells were prepared as described for real-time PCR analysis above. Cells were lysed using 1x SDS-lysis buffer. The cell lysis was centrifuged at 10,000 g for 10 min and the supernatant was collected for Western Blot analysis. In short, protein lysates and conditioned medium was loaded on 10% Tris-Glycine gels (Thermo Scientific) and transferred onto PVDF membranes (Thermo Scientific). The membranes were incubated overnight at 4°C with the required primary antibody (Table 3), followed by incubation with species specific horseradish peroxidase (HRP) conjugated secondary and tertiary antibody for 1 hour at RT. HRP was detected with Pierce™ ECL Plus Western Blotting Substrate kit (Thermo Scientific) and exposed to FluorChem™ M System (ProteinSimple, San José, USA). The protein levels were quantified by Image J Software (NIH, MD).

Table 3. Primary and secondary antibodies used for western blot.

Antibody	Source	Dilution
Goat-anti-Integrin α 11	R&D Systems	1:250
Mouse anti- α -SMA	Sigma Aldrich	1:200
Mouse monoclonal anti- β -actin	Sigma Aldrich	1:10000
HRP-conjugated goat anti-rabbit IgG	DAKO	1:2000
HRP-conjugated rabbit anti-goat IgG	DAKO	1:2000
HRP-conjugated rabbit anti-mouse IgG	DAKO	1:1000

6.2.6 F-actin staining

Cells were prepared as described above for quantitative real-time PCR. Subsequently the cells were fixed in PBS containing 10% formaldehyde (Sigma Aldrich) for 15 min. After permeabilization with 0.1 M Triton X-100 (Sigma Aldrich) for 5 min, F-actin was stained with phalloidin (Life Technologies, Carlsbad, USA) at a concentration of 250 ng/ml for 30 minutes at RT. Next, cells were washed with PBS and imaged for phalloidin at (ex. 540/ em. 565) using an EVOS FI fluorescent microscope (Life Technologies).

6.2.7 Immunocytochemistry

Cells were prepared as described above for quantitative real-time PCR. Next, cells were fixed and immunostained for α -SMA, and Collagen-1 as described elsewhere ²⁵.

6.2.8 Cell proliferation assay

hPSCs were seeded at a density of 2,500 cells/well into a 96-well plate, starved for 24 h and activated with 5 ng/ μ l TGF- β or Panc-1 conditioned medium. Cell number was monitored every 24 h over a time period of 72 h. To measure the cell number, 10 μ l of Alamar Blue dye (Invitrogen, Carlsbad, USA) in 100 μ l of hPSC growth media was added per well. The fluorescent signal was measured after incubation for 4 h at 37 °C using a VIKTOR™ plate reader (Perkin Elmer, Waltham, Massachusetts).

6.2.9 3D-Collagen I Gel Contraction Assay

A collagen suspension consisting of 3.0 ml Collagen G1 (5 mg/ml, Matrix biosciences, Morlenbach, Germany), 0.5 ml 10x M199 medium (Sigma Aldrich), 85 μ l 1N NaOH (Sigma Aldrich) and sterile water was mixed with 1.0 ml of cell suspension containing 200,000 shCTR or shITGA11 hPSCs. Per well of a 24 well plate 600 μ l of collagen gel / cell was plated and allowed to form a gel for 1 h at 37 °C. Once the collagen gel was formed, 1 ml of serum free medium was added with or without TGF β (5 ng/ml) or Panc-1 conditioned medium (CM). Next, the gels were detached from the walls of the well plate with a 10 μ l pipette tip. Representative images were made at 72 h using a digital camera. Measurement of collagen gel diameter were normalized with the diameter of the respective well and performed using Image J imaging software (NIH, Bethesda, MD).

6.2.10 Conditioned Media

To collect hPSC conditioned medium, shCTR and shITGA11 hPSCs were seeded into a 12 well plate at a seeding density of 40.000 cells/ well. As previously described, cells were starved and activated with TGF- β or Panc-1 conditioned medium for 24 h. Next, the cells were washed 3 times with serum-free medium. After 48 h of incubation with serum-free medium, the medium was collected for use in conditioned medium experiments.

6.2.11 Wound Healing Assay

Cells were seeded into a 24-well plate at a seeding density of 50.000 cells/well. Cells were starved and TGF- β activated as described previously. To study migration, a scratch was made on the culture plate using a 200 μ l pipette tip fixed in a custom-made holder. The cells were washed to remove all detached cells and incubated with fresh serum-free media. Images were captured at $t = 0$ h and $t = 12$ h, with an EVOS microscope. Images were analyzed by Image J software to calculate the area of the scratch and represented as the percentage of wound closure.

6.2.12 Transwell Migration Assay

Panc-1 cells were seeded at a density of 50.000 cells/well into the upper chamber of a 24 well 8 μ m transwell insert (Sigma Aldrich). The lower compartment of the transwell insert was filled with conditioned medium from shITGA11 and shCTR hPSCs as chemoattractant. After 24 h, the cells were fixed with ice cold methanol (Fisher Scientific) for 10 min and incubated with 0.1 % Crystal Violet solution (Sigma Aldrich) in 25% methanol (Fisher Scientific) for 10 min. After washing the cells with MilliQ water, cells from the upper compartment were removed with a cotton swap. After drying the inserts the migrated cells were imaged with a Nikon microscope (Nikon Eclipse E400, Tokyo, Japan). The positively stained area per picture was analyzed by Image J software.

6.2.13 Transwell Invasion Assay

The upper and lower compartment of 8 μ m BD Matrigel™ Invasion Chambers (BD Biosciences, Bedford, USA) were filled with warm DMEM containing 0% FBS and rehydrated for 2 h at 37°C. Next the DMEM was removed from both compartments and Panc-1 cells were seeded at a density of 50.000 cells/well into the upper compartment of the Invasion Chamber. The lower compartment of the transwell insert was filled with conditioned medium from shITGA11 and shCTR hPSCs as chemoattractant. After 48 h, the gel from the upper compartment was removed with a cotton swap. Next, the cells were fixed with ice cold methanol

(Fisher Scientific) for 10 min and incubated with 0.1 % Crystal Violet solution (Sigma Aldrich) in 25% methanol (Fisher Scientific) for 10 min and subsequently washed with MilliQ water. After drying the inserts the invaded cells were imaged with a Nikon microscope (Nikon Eclipse E400, Tokyo, Japan). The positively stained area per picture was analyzed by Image J software.

6.2.14 Cytokine proteome profiler protein array

The conditioned medium to be tested in the cytokine array was collected as described previously. The RayBio® C-Series Human Cytokine Array C5 was performed as described by the manufacturer. Cytokine array membranes were imaged using a FluorChem™ M System (ProteinSimple). The protein levels were quantified by Image J Software (NIH, MD).

6.3 Results

6.3.1 ITGA11 is specifically expressed on CAFs in human PDAC

Immunohistochemical analyses for ITGA11 expression in human PDAC sections showed a clear ITGA11 expression in tumor stroma while no expression in the tumor epithelial cells. In addition, ITGA11 was completely absent in normal human pancreatic tissues (Figure 1 A). Visual scoring (n=6 patients) showed that 66.7 % of stained tumors showed a moderate expression of ITGA11 in the tumor stroma while 33.3 % showed a high stromal expression. Additionally, we examined the expression of the well-known CAF marker α -SMA in combination with ITGA11 in PDAC sections (Figure 1 B). Scoring of the co-localization of these two markers showed that 80 % of α -SMA positive cells were also positive for ITGA11, 20 % of α -SMA positive cells were negative for ITGA11, while only 5% of ITGA11 positive cells were negative for α -SMA (Figure 1 B).

6.3.2 TGF- β induces ITGA11 expression in hPSCs

To determine whether ITGA11 expression is specifically induced in primary human activated hPSCs, we compared the gene and protein expression of ITGA11 in TGF- β -activated and non-activated control hPSCs. We found that ITGA11, on both gene and protein level, expressed only in low levels in non-activated hPSCs, while its expression levels were induced in TGF- β -activated cells (Figure 2 A, B). Also, the induction of ITGA11 was in line with hPSC activation and the CAF markers, collagen-I (Col1a1) and α -SMA, (Figure 2 C,

D). Additionally, no expression of ITGA11 was found in the human pancreatic cancer cell lines Panc-1, MiaPaCa-2 and AsPc-1 (Figure 2 B).

Figure 1. *ITGA11 is specifically expressed on CAFs in human PDAC. A) Staining of human PDAC and normal pancreas for ITGA11. Scale bar = 250 μ m. B) Co-localization of ITGA11 with α -SMA. Scale bar = 100 μ m.*

Figure 2. TGF- β induces ITGA11 expression in hPSCs. A) Gene expression and B) protein expression of ITGA11 in TGF- β -activated and non-activated hPSCs. C) Gene expression and D) protein expression of α -SMA and Col1a1 in TGF- β -activated and non-activated hPSCs. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.d. stands for not detected.

6.3.3 Knockdown of ITGA11 inhibits TGF- β mediated differentiation of hPSCs

To demonstrate the role for ITGA11 in hPSC activation, we stably knocked down ITGA11 in hPSCs using shRNA plasmid and evaluated the effect of the knockdown on the hPSC phenotype and TGF- β -mediated activation and differentiation. The knockdown of ITGA11 was confirmed using real time qPCR and western blot analysis, showing a reduction of about 50% at the mRNA level and about 85% at the protein level in ITGA11 knockdown (shITGA11) hPSC compared to the control (shCTR) hPSCs (Figure 3 A, B). ITGA11 knockdown resulted in a drastic phenotypic change leading to cellular hypertrophy compared to shCTR hPSCs (Figure 3 C). Upon activation with TGF- β , both shCTR and shITGA11 hPSCs showed only a slight induction in ITGA11 levels (Figure 3 D). Surprisingly, the induction of ITGA11 and α -SMA protein levels with TGF- β

was less in shCTR hPSCs when compared with normal hPSCs (Figure 2B), which can be attributed to higher passage numbers which was unavoidable to achieve stable transfection. Strikingly, TGF- β -mediated induction of α -SMA in shITGA11 hPSCs was significantly attenuated compared to shCTR hPSCs (Figure 3D). Immunostaining showed the same trend for the protein expression of Col1a1 and α -SMA (Figure 3E).

Additionally, we examined the effect of ITGA11 knockdown on CAF marker and found that ITGA11 knockdown significantly downregulated the gene expression of CAF markers such as Platelet derived growth factor β receptor (PDGF β R), Desmin, Collagen 3 (Col3a1), NAD(P) Dependent Steroid Dehydrogenase-Like (NSDHL), Insulin-induced gene 1 (INSIG1), Isopentenyl-diphosphate Delta-isomerase 1 (IDI1) and C-X-C chemokine receptor type 4 (CXCR-4) (Figure 3F). Upon activation with TGF- β , shITGA11 hPSCs showed an attenuated expression in the CAF markers PDGF β R, Desmin, NSDHL, INSIG1, IDI1, MMP-2 and CXCR-4 as compared to shCTR hPSCs (Figure 3E), indicating that these genes are regulated by ITGA11. We further analyzed shCTR and shITGA11 hPSCs with and without TGF- β -mediated activation for the mRNA gene expression profiling of 84 ECM and adhesion proteins using a human profiler gene array. Genes downregulated after the knockdown of ITGA11 included various receptors responsible for the adhesion to immune cells (SELE, ITGB2, SELP, ITGAL, SELL, ITGAM), laminin receptors (ITGA7, ITGB4), collagen receptors (ITGA8), ECM molecules (COL11A1, ECM1, LAMA1, VTN), MMPs and TIMPs (MMP8, MMP9, MMP10, MMP11, MMP12, MMP13 and TIMP3), cell adhesion molecules (CNTN1, CDH1, CTNND2, THBS2, CLEC3B, NCAM1) and a protein involved in ECM production (HAS1) (Table 4). Furthermore, we identified TGF- β -induced genes which are regulated by ITGA11. We found several genes whose expression was not induced in shITGA11 hPSC but in shCTR hPSC after activation with TGF- β (Table 5). These included cell adhesion molecules (THBS1, SPP1), growth factors (CTGF), ECM molecules (TNC, FN1, COL8A1, TGFBI, COL5A1), MMPs and TIMPs (TIMP1, MMP3) and protein involved in ECM production (SPARC).

Figure 3. Knockdown of ITGA11 inhibits TGF- β mediated differentiation of hPSCs. A) Real time qPCR and B) Western Blot the shRNA mediated knockdown of ITGA11. C) F-actin staining of shCTR compared to shITGA11 hPSCs. Scale bar = 100 μ m. D) Western blot for ITGA11 and α -SMA of shCTR and shITGA11 hPSCs with and without

*TGF- β activation. E) Immunostaining for α -SMA and Col1a1 of shCTR and shITGA11 hPSCs with and without TGF- β activation. Scale bar = 200 μ m. F) Real time qPCR for the CAF markers α -SMA, PDGF β R, Desmin, Col3a1, NSDHL, INSIG1, IDI1 and CXCR-4. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Table 4. Genes downregulated in shITGA11 hPSCs compared with shCTR hPSCs with a fold regulation ≤ -2 . The data was obtained with the RT² Profiler™ PCR Array Human Extracellular Matrix & Adhesion Molecules (Qiagen) and analyzed using the data analysis center provided by Qiagen.

Gene ID	Refseq	Description	Fold Regulation
SELE	NM_000450	Selectin E	-11,26
CNTN1	NM_001843	Contactin 1	-9,31
TIMP3	NM_000362	TIMP metalloproteinase inhibitor 3	-7,25
CDH1	NM_004360	Cadherin 1	-5,93
ITGA7	NM_001144996	Integrin subunit alpha 7	-5,57
MMP8	NM_002424	Matrix metalloproteinase 8	-4,89
CTNND2	NM_001332	Catenin delta 2	-4,59
ITGB4	NM_000213	Integrin subunit beta 4	-4,57
HAS1	NM_001523	Hyaluronan synthase 1	-4,49
ITGB2	NM_000211	Integrin subunit beta 2	-4,42
THBS2	NM_003247	Thrombospondin 2	-3,68
COL11A1	NM_001854	Collagen type XI alpha 1 chain	-3,38
SELP	NM_003005	Selectin P	-3,33
ECM1	NM_004425	Extracellular matrix protein 1	-3,04
ITGAL	NM_002209	Integrin subunit alpha L	-2,96
MMP10	NM_002425	Matrix metalloproteinase 10	-2,67
MMP11	NM_005940	Matrix metalloproteinase 11	-2,65
SELL	NM_000655	Selectin L	-2,63
MMP9	NM_004994	Matrix metalloproteinase 9	-2,57
CLEC3B	NM_003278	C-type lectin domain family 3 member B	-2,52
LAMA1	NM_005559	Laminin subunit alpha 1	-2,51
ITGAM	NM_001145808	Integrin subunit alpha M	-2,45
MMP12	NM_002426	Matrix metalloproteinase 12	-2,44
ITGA8	NM_003638	Integrin subunit alpha 8	-2,41
VTN	NM_000638	Vitronectin	-2,34
MMP13	NM_002427	Matrix metalloproteinase 13	-2,21

NCAM1 NM_000615 Neural cell adhesion molecule 1 -2,03

Table 5. Genes upregulated in TGF β -activated shCTR and shITGA11 hPSCs compared with their respective control. The data was obtained with the RT² Profiler™ PCR Array Human Extracellular Matrix & Adhesion Molecules (Qiagen) and analyzed using the data analysis center provided by Qiagen.

			shCTR + TGFβ	shITGA11 + TGFβ	
Gene ID	Refseq	Description	Fold Regulation		Δ Difference
THBS1	NM_003246	Thrombospondin 1	14,26	3,32	10,94
CTGF	NM_001901	Connective tissue growth factor	6,22	2,81	3,41
TNC	NM_002160	Tenascin C	7,65	5,92	1,73
TIMP1	NM_003254	TIMP metalloproteinase inhibitor 1	2,49	1,06	1,43
MMP3	NM_002422	Matrix metalloproteinase 3	2,41	1,43	0,98
SPP1	NM_001251830	Secreted phosphoprotein 1	2,71	1,82	0,89
FN1	NM_212474	Fibronectin 1	2,28	1,73	0,55
COL8A1	NM_001850	Collagen type VIII alpha 1 chain	2,47	1,96	0,51
SPARC	NM_003118	Secreted protein acidic and cysteine rich	2,16	1,86	0,3
TGFB1	NM_000358	Transforming growth factor beta induced	2,25	1,96	0,29

6.3.4 Knockdown of ITGA11 inhibits Panc-1 conditioned medium mediated differentiation of hPSCs

We investigated the influence of Panc-1 conditioned medium on ITGA11 expression and Panc-1 conditioned medium induced activation of hPSCs and shITGA11 hPSCs. Unlike TGF- β treatment, treatment of hPSCs with Panc-1 CM did not induce ITGA11 levels in either shITGA11 or shCTR hPSCs (Figure 4 A). The protein expression of α -SMA was induced in shCTR hPSCs activated with Panc-1 CM, while these inductions were attenuated in shITGA11 hPSCs (Figure 4 A). Immunostaining showed the same trend for the protein expression of Colla1 and α -SMA (Figure 4 B).

Figure 4. Knockdown of ITGA11 inhibits Panc-1 conditioned medium mediated differentiation of hPSCs. A) Western blot for ITGA11 and α-SMA of shCTR and shITGA11 hPSCs with and without Panc-1 CM activation. B) Immunostaining for α-SMA and Col1a1 of shCTR and shITGA11 hPSCs with and without Panc-1 CM activation. Scale bar = 200 μm. C) Real time qPCR of the CAF markers α-SMA, PDGFR, Desmin, Col3a1, NSDHL, INSIG-1, IDI1 and CXCR-4. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Analysis of the gene expression of well-known CAF markers in shITGA11 revealed significant downregulation of PDGF β R, Desmin, Col3a1, NSDHL, INSIG-1, IDI-1 and CXCR-4 as compared to shCTR hPSCs (Figure 4 C). When these cells were activated with Panc-1 CM, the CAF markers α -SMA, PDGF β R, Desmin, INSIG-1 and CXCR-4 showed an attenuated expression in shITGA11 as compared to shCTR hPSCs (Figure 4 C). It needs to be noted, that among these markers only PDGF β R was significantly induced with Panc-1 CM (Figure 4 C).

6.3.5 Knockdown of ITGA11 attenuates TGF- β induced hPSC function

We furthermore investigated whether ITGA11 regulates the functions of hPSCs including migration and contraction. Human PSCs are known for their ability to migrate to the tumor site and differentiate into CAFs with high contractile properties and form metastasis ²⁶.

Figure 5. Knockdown of ITGA11 attenuates TGF- β induced hPSC function. A) Representative images and quantification of collagen gel contraction of shCTR and shITGA11 hPSCs with and without TGF- β activation. B) Representative microscopic images and quantification showing the migration of shCTR and shITGA11 hPSCs with and without TGF- β activation. Scale bar = 500 μ m. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * p < 0.05, ** p < 0.01, *** p < 0.001.

While non-activated hPSCs do not have the ability to contract, hPSCs activated with TGF- β acquire a contractile phenotype. In comparison to shCTR hPSCs, shITGA11 hPSCs showed significantly poor contractile ability (Figure 5 A). Furthermore, cell migration (with and without TGF β induction) was attenuated in shITGA11 hPSCs as compared to shCTR hPSCs (Figure 5 B).

6.3.6 Knockdown of ITGA11 attenuates Panc-1 CM induced hPSC function

Next to TGF- β , we also investigated the effect of ITGA11 knockdown in hPSCs on functional activation with Panc-1 CM. Similar to TGF- β activation, the activation with Panc-1 CM led to the induction of the contractility of shCTR hPSCs and these inductions were diminished in shITGA11 hPSCs (Figure 6 A). In contrast to TGF β activation, Panc-1 CM did not show induction of migration of shCTR hPSC but yet ITGA11 knockdown reduced the migration of hPSCs (Figure 6 B). These data indicate that ITGA11 is able to regulate the activation of hPSCs by controlling more pathways than only TGF- β .

Figure 6. Knockdown of ITGA11 attenuates Panc-1 CM induced hPSC function. A) Representative images and quantification of collagen gel contraction by shCTR and shITGA11 hPSCs with and without Panc-1 CM activation. B) Representative microscopic images and quantification showing the migration of shCTR and shITGA11 hPSCs with and without Panc-1 CM activation. Scale bar = 500 μ m. Data represent

mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. ** $p < 0.01$, *** $p < 0.001$.

6.3.7 Effect of ITGA11 on the secretome of hPSCs

Furthermore, we analyzed the effect of ITGA11 knockdown on the secretome of hPSCs, which is crucial for the crosstalk with tumor cells and other cells in the tumor microenvironment. We found a decrease or increase in the secretion of several cytokines and growth factors by shITGA11 hPSCs compared to shCTR hPSCs (Supplementary Table 1, Figure 7).

Figure 7. Effect of ITGA11 on the secretome of hPSCs. A) Results from the Human Cytokine Antibody Array C5 (RayBio®) for shCTR and shITGA11 hPSC, without and with activation with TGF- β or Panc-1 CM. Boxes indicate internal positive controls. B) Map pf the Human Cytokine Antibody Array C5.

The top five downregulated and upregulated cytokines and growth factors are shown in Table 6. Additionally, we found that the growth factors MIF, TGF- β 3, IGFBP-1, IGFBP-3, CXCL1, PDGF-BB, FGF-6, FGF-7, CCL22 and CCL23 were induced by TGF- β in shCTR hPSCs, while their expression was attenuated in shITGA11 hPSCs treated with TGF- β (Table 7). On the contrary, we found

LIF, CCL4 and M-CSF to be induced to a higher extent in shITGA11 compared to shCTR hPSCs upon treatment with TGF- β (Table 7, Figure 7).

Table 6. Top 10 cytokines and growth factors downregulated or upregulated in shITGA11 hPSCs compared with shCTR. The data was obtained with the Human Cytokine Antibody Array C5 (RayBio®) and quantified using ImageJ. Blot of the Human Cytokine Antibody Array C5 can be found in figure 7.

Protein ID	Description	Fold Regulation
M-CSF	Macrophage colony-stimulating factor	-2,87
Angiogenin	Angiogenin	-2,09
CCL4	C-C motif chemokine 4	-1,89
TGF-b 3	Transforming growth factor beta 3	-1,83
CCL22	C-C motif chemokine 22	-1,83
FGF-6	Fibroblast growth factor 6	1,89
CCL2	C-C motif chemokine 2	2,06
MIF	Macrophage migration inhibitory factor	2,15
OPG	Osteoprotegerin	2,49
IL-6	Interleukin-6	3,06

Table 7. Top cytokines and growth factors with a differential expression in TGF- β -activated shCTR and shITGA11 hPSCs. Only cytokines and growth factors which showed ≥ 1.1 fold-upregulation with Panc-1 CM in shCTR hPSCs and a Δ difference ≥ 0.1 are shown. The data was obtained with the Human Cytokine Antibody Array C5 (RayBio®) and analyzed using ImageJ. Blot of the Human Cytokine Antibody Array C5 can be found in figure 7.

		shCTR + TGF-β	shITGA11 + TGF-β	
Protein ID	Description	Fold Regulation		Δ Difference
MIF	Macrophage migration inhibitory factor	1,24	-1,20	2,44
TGF-b 3	Transforming growth factor beta 3	1,19	-1,18	2,37
IGFBP-1	Insulin-like growth factor-binding protein 1	1,32	-0,84	2,16
CXCL1	Growth-regulated alpha protein	1,20	-0,88	2,07
PDGF-BB	Platelet-derived growth factor subunit B	1,69	-0,38	2,07
IGFBP-3	Insulin-like growth factor-binding protein 3	1,71	-0,24	1,95
FGF-6	Fibroblast growth factor 6	1,58	-0,32	1,90

CCl23	C-C motif chemokine 23	1,23	-0,60	1,83
FGF-7	Fibroblast growth factor 7	1,45	1,19	0,26
CCL22	C-C motif chemokine 22	4,33	4,13	0,20
LIF	Leukemia inhibitory factor	1,25	1,67	-0,41
CCL4	C-C motif chemokine 4	1,83	2,61	-0,79
M-CSF	Macrophage colony-stimulating factor	2,05	3,12	-1,07

As shown previously by others, conditioned medium (CM) of Panc-1 tumor cells contains a mixture of different cytokines, like IL-8, IGFBP-1, OPG, VEGF, Angiogenin, IGFBP-2, PIGF and GM-CSF 27. We evaluated whether knockdown of ITGA11 in hPSCs not only attenuated TGF- β -induced secretions, but also Panc-1 condition medium (CM)-induced secretion from hPSCs. We found that the secreted factors CXCL1, IGFBP-1 and PDGF-BB were induced by Panc-1 CM in shCTR hPSCs, while their expression was attenuated in shITGA11 hPSCs treated with Panc-1 CM (Table 8, Figure 7). On the contrary, we found IGFBP-3 and CCL-2 to be more induced in shITGA11 compared to shCTR hPSCs upon treatment with Panc-1 CM (Table 8, Figure 7).

Table 8. Differential expression of Panc-1 CM-induced cytokines and growth factors in shCTR and shITGA11 hPSCs. Only cytokines and growth factors which showed ≥ 1.1 fold-upregulation with Panc-1 CM in shCTR hPSCs and a Δ difference ≥ 0.1 are shown. The data was obtained with the Human Cytokine Antibody Array C5 (RayBio®) and analyzed using ImageJ. Blot of the Human Cytokine Antibody Array C5 can be found in figure 7.

Protein ID	Description	shCTR + Panc-1 CM	shITGA11 + Panc-1 CM	Δ Difference
		Fold Regulation		
CXCL1	Growth-regulated alpha protein	1,10	-1,07	2,17
IGFBP-1	Insulin-like growth factor-binding protein 1	1,44	-0,62	2,07
PDGF-BB	Platelet-derived growth factor subunit B	1,14	-0,36	1,51
IGFBP-3	Insulin-like growth factor-binding protein 3	1,18	1,61	-0,43
CCL22	C-C motif chemokine 22	1,47	2,74	-1,27

6.3.8 Conditioned medium from hPSCs with knockdown of ITGA11 inhibits paracrine effects on Panc-1

After confirming the direct effects of ITGA11 knockdown on hPSC activation, we investigated whether ITGA11 knockdown is able to inhibit hPSC-mediated paracrine effects on tumor cell migration and invasion.

Figure 8. Conditioned medium from hPSCs with knockdown of ITGA11 inhibit paracrine effects on Panc-1. A) Representative images and quantification of Panc-1 tumor cell migration after 24 hours of incubation with conditioned medium obtained from shCTR and shITGA11 hPSCs with and without TGF- β activation. Scale bar = 400 μ m. B) Representative images and quantification of Panc-1 tumor cell invasion after 24 hours of incubation with condition medium collected from shCTR and shITGA11 hPSCs with and without TGF- β activation. Scale bar = 400 μ m. Data represent mean + SEM for at least 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student t-test. * $p < 0.05$.

To evaluate this, we collected conditioned medium from non-activated and TGF- β activated shCTR and shITGA11 hPSCs. We found that Panc-1 cells incubated

with CM from shITGA11 hPSCs had a reduced ability to migrate (Figure 8 A) and invade (Figure 8 B) in transwell inserts.

6.4 Discussion

The present study introduces the collagen 1 binding transmembrane receptor ITGA11 as a significant regulator of the CAF phenotypic differentiation and function. ITGA11 was shown to be substantially expressed in the stroma of human PDAC and co-localized with the major CAF marker α -SMA in human patient samples. Strikingly, the knockdown of ITGA11 in hPSCs inhibited their differentiation, migration and contractility. A profiler array showed that many ECM and adhesion molecules were downregulated in hPSCs after ITGA11 knockdown. Moreover, our data revealed that the knockdown of ITGA11 attenuated hPSCs mediated paracrine effects on Panc-1 tumor cells.

The pancreatic tumor stroma is well known as one of the hallmarks of PDAC. CAFs, the most abundant stromal cells, promote PDAC progression by producing high amounts of ECM and secreting various cytokines and growth factors, which in turn stimulate tumor growth, angiogenesis, invasion, and metastases^{5-7,27}. Human PSCs are regarded as the main precursors of CAFs in the pancreatic tumor stroma¹⁶ and in response to TGF- β they acquire a CAF-like myofibroblast phenotype, as we and others have shown previously²⁸⁻³⁰. Therefore, modulating the tumor stroma through therapeutic strategies which aim to inhibit the activation of hPSCs into CAFs is a rational approach to gain therapeutic benefits for the treatment of PDAC. ECM transmembrane receptors of the integrin family, allow cells to attach, migrate and proliferate. Some integrin receptors are strongly upregulated in CAFs, which by themselves produce high amounts of ECM, including collagens¹⁸. Integrin targeting in myofibroblasts has been shown to inhibit fibrosis in multiple organs such as liver, lung and kidney^{18,24,31,32}.

In the present study, we show that ITGA11 is highly upregulated in the tumor stroma of human PDAC. Co-immunostaining of ITGA11 with α -SMA showed that ITGA11 was almost exclusively expressed on CAFs within these tissues, with 80% of α -SMA positive cells being ITGA11 positive and only 20 % of α -SMA positive cells being ITGA11 negative. Interestingly, no expression of ITGA11 was found in healthy pancreatic tissue, suggesting that therapeutic targeting of this receptor is likely to have a minimal side effects on normal tissues.

We found that quiescent hPSCs had an extremely low expression of ITGA11, while activation with TGF- β resulted in a significant upregulation of ITGA11, which correlated with the significant upregulation of the main CAF markers α -SMA and Colla1. The induction of ITGA11 can be attributed to the TGF- β mediated regulation of ITGA11 in a Smad Sp-1 dependent manner which was previously demonstrated ³³. Stable knockdown of ITGA11 in hPSCs significantly attenuated the TGF- β and Panc-1 CM mediated differentiation of hPSCs and ECM production as shown with the CAF related markers α -SMA and Colla1 (Figure 3, 4). In addition, ITGA11 knockdown significantly reduced the TGF- β and Panc-1 CM-induced expression of PDGF β R, Desmin, NSDHL, IDI-1, INSIG-1, MMP-2 and CXCR-4. These markers were previously found to be induced in activated PSCs by transcriptome analysis ^{8,34}. Of high significance is the downregulation of ECM proteins e.g. collagens limiting chemotherapeutic activity by blocking drug delivery ^{35,36}. In addition, the gene profiler array for ECM and cell adhesion molecules revealed genes which were controlled by ITGA11, as shown by the knockdown of ITGA11. Among them, selectin, connectin, and TIMP3 were the top downregulated genes. Furthermore, we also revealed TGF- β -induced genes regulated by ITGA11 in hPSCs, and among them thrombospondin and CTGF, known to decrease the tumor response to gemcitabine ³⁷, were the most reduced ones (Table 5).

We further studied whether ITGA11 controls the function of hPSCs. Interestingly, ITGA11 knockdown resulted in a loss of cell-contraction or migration, with and without TGF- β or Panc-1 CM mediated activation, of hPSCs. Overall, these data demonstrated that ITGA11 is crucial for the maintenance of the phenotypic features and activation of hPSCs.

Since hPSCs crosstalk with tumor cells and other stromal cells in the tumor microenvironment, we examined the effect of ITGA11 on the secretome of hPSCs. It has previously been demonstrated that hPSCs can induce tumor-promoting paracrine effects by secreting mutagenic factors such as growth factors and cytokines to induce tumor progression, invasion and metastasis ^{5,38}. In the present study, the knockdown of ITGA11 in quiescent hPSCs results in a phenotype with a reduced but also induced expression of tumor promoting factors. For example M-CSF, the most downregulated factor, is a cytokine which is known to be secreted by CAFs and promote the tumor-associated macrophage phenotype of monocytes in PDAC ³⁹. However, IL-6, the most upregulated factor, was shown to promote pancreatic cancer invasiveness (?) ⁴⁰. Upon activation of hPSCs with either TGF- β or Panc-1 CM, various cytokines (CCL-

22, PDGF-BB, MIF, IGFBP-1) were induced in shCTR hPSCs. Although not all the induced cytokines were controlled by ITGA11, there were a number of cytokines (MIF, IGFBP-1, CXCL1, TGF- β 3) whose secretion was reduced in shITGA11 hPSCs. Some of these factors are highly interesting in the context of pancreatic cancer progression. CXCL-1 has been identified to transform healthy fibroblasts into CAFs in an autocrine manner⁴¹, contributes to tumor progression by mediating interactions between cancer cells, tumor-associated macrophages and CAFs⁴² and contributes to radioresistance⁴³. TGF- β 3 is known induce growth, migration and invasion on cancer cells⁴⁴. PDGF-BB promoted stroma formation and tumor growth⁴⁵. Interestingly, we also found that conditioned medium from shITGA11 hPSCs showed an attenuated migration and invasion of Panc-1 tumor cells compared to shCTR hPSCs. This might be due to TGF- β 3, known to induce the migration and invasion of cancer cells⁴⁴, which is reduced in the conditioned medium of shITGA11 compared to shCTR hPSCs and is only induced with TGF- β in shCTR but not in shITGA11 hPSCs.

Next to the reduced secretion of certain cytokines, there were also cytokines which were induced in shITGA11 hPSCs. However, with the exception of IGFBP-3, these cytokines were in the top six of cytokines found to be downregulated by ITGA11 knockdown, therefore their higher induction in TGF- β or Panc-1 CM activated shITGA11 compared to shCTR hPSCs might be due to a compensatory cellular mechanism, which re-induces the expression of these factors.

In conclusion, the present study for the first time demonstrates the expression and role of ITGA11 in pancreatic tumor stroma. ITGA11 was specifically and strongly expressed in the tumor stroma co-localized with CAFs. Furthermore, we show ITGA11 as a crucial target in maintenance of the phenotype and activation and differentiation of hPSCs. Nonetheless, our knockdown studies demonstrated a direct regulatory effect of ITGA11 on the transcriptome and secretome of activated hPSCs which controls their paracrine function. Altogether, ITGA11 is a promising therapeutic target for developing new therapeutics to inhibit the hPSC-mediated crosstalk in the tumor microenvironment.

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6.6 Conflicts of interest

J Prakash is a founder and stakeholder of ScarTec Therapeutics BV. Enschede. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

6.7 Reverences

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6.8 Supplementary

Supplementary Table 1. Cytokines and growth factors downregulated or upregulated in *shITGA11* hPSCs compared with *shCTR*. The data was obtained with the Human Cytokine Antibody Array C5 (RayBio®) and quantified using ImageJ.

Protein ID	Description	Fold Regulation
M-CSF	Macrophage colony-stimulating factor	-2,874093954
Angiogenin	Angiogenin	-2,088758183
CCL4	C-C motif chemokine 4	-1,890804333
TGF-b 3	Transforming growth factor beta 3	-1,832072346

CCL22	C-C motif chemokine 22	-1,825996084
LIF	Leukemia inhibitory factor	-1,539654607
VEGF-A	Vascular endothelial growth factor A	-1,455564159
TNF beta	Tumor necrosis factor	-1,360741111
GRO a/b/g	Oncogene GRO a/b/g	-1,275876741
BDNF	Brain-derived neurotrophic factor	-1,183278706
HGF	Hepatocyte growth factor	-1,15222553
TIMP-1	Tissue inhibitor of metalloproteases	-1,110709638
CCL-17	C-C motif chemokine ligand 17	-1,080764946
IP-10	C-X-C motif chemokine 10	-1,061076338
OSM	Oncostatin-M	-1,043949116
EGF	Epidermal growth factor	-1,021746053
TIMP-2	Metalloproteinase inhibitor 2	1,019660243
IL-8	Interleukin-8	1,030393099
PDGF-BB	Platelet-derived growth factor subunit B	1,143836841
IGFBP-2	Insulin-like growth factor-binding protein 2	1,204848312
CXCL5	C-X-C motif chemokine 5	1,223342142
TGF-b 2	Transforming growth factor beta 2	1,238059015
IGFBP-3	Insulin-like growth factor-binding protein 3	1,24913089
CCl23	C-C motif chemokine 23	1,311194625
IL-7	Interleukin-7	1,33322543
OPN	Osteopontin	1,564699424
CXCL1	C-X-C motif chemokine 1	1,669274325
FGF-9	Fibroblast growth factor 9	1,706309402
IGF-1	Insulin-like growth factor I	1,730488364
FGF-7	Fibroblast growth factor 7	1,731072065
IGFBP-1	Insulin-like growth factor-binding protein 1	1,758058869
FGF-6	Fibroblast growth factor 6	1,891364755
CCL2	C-C motif chemokine 2	2,05651861
MIF	Macrophage migration inhibitory factor	2,149189951
OPG	Osteoprotegerin	2,486250312
IL-6	Interleukin-6	3,061058772

Chapter 7 (page 199-224) under embargo until 31-12-2023

Chapter 8 - Summary and Discussion

Pancreatic ductal adenocarcinoma (PDAC) is the cancer type with the worst prognosis. The 1-year survival rate is 20%, whereas the 5-year survival rate is 8%¹. A surgical resection is only feasible in a small percentage of PDAC tumors, making radiotherapy or cytotoxic chemotherapy the golden standard treatment. A lot of research has been conducted in search of more effective therapies for PDAC. However, only marginal improvements have been made with regard to the survival rate²⁻⁴. The abundant desmoplasia in PDAC, also called the tumor stroma or tumor microenvironment, which supports cancer progression, invasion and metastasis, is one of the main causes for treatment failure⁵.

Abundant components of the tumor stroma include cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), pericytes, endothelial cells, infiltrating immune cells (e.g. T-cells, natural killer (NK) cells and macrophages) and ECM proteins (e.g. collagen)⁶. CAFs are the key drivers of the pancreatic tumor stroma, with their main precursors, human pancreatic stellate cells (hPSCs)⁶⁻⁸. Quiescent hPSCs are known for their vitamin A storing ability, can be found within the connective tissue of the pancreas in low numbers and secrete only small amounts of extracellular matrix (ECM) proteins (e.g. collagen)⁹. During PDAC progression, quiescent hPSCs become activated into CAF-like myofibroblasts as response to tissue injury and growth factors such as TGF- β 1. As a result, they lose their cytoplasmic lipid storing capacity and start secreting high amounts of ECM components which leads to desmoplasia. Desmoplasia, fibrotic stromal tissue, acts as a physical barrier, preventing drug accumulation in the tumor tissue^{10,11}. Additionally, activated hPSCs secrete various cytokines and growth factors, which stimulate tumor cells, thereby facilitating tumor growth, invasion and metastasis and ultimately tumor progression^{12,13}. Therefore, modulation of the tumor stroma by dampening the function of activated hPSCs presents an innovative approach to gain therapeutic benefits and improve the treatment of PDAC.

In this thesis, we focused on the identification of new therapeutic targets, including microRNA (miRNA), integrin receptors and bioactive lipids, to reprogram activated hPSCs into their quiescent phenotype and thereby modulate the tumor stroma in pancreatic cancer. Furthermore, we designed a novel system for miRNA delivery into hPSC and designed an integrin alpha 11 specific peptide for targeting of drug delivery systems to activated hPSCs in the stroma of PDAC.

In **chapter 2**, we introduce miRNAs in the tumor stroma, which have emerged as key players involved in the development of cancer and its progression. MicroRNAs present a potential class of therapeutics, which regulate various signaling pathways within the tumor microenvironment ¹⁴. In general, miRNAs are a class of small (17–25 nt), endogenous, single stranded non-coding RNAs, which control gene expression at the post-transcriptional level and thereby control various cellular processes ¹⁵. A unique ability of miRNAs is their ability to regulate not only one but hundreds of target genes simultaneously, enabling them to control multiple signaling pathways ¹⁶. This is possible, because miRNAs silence genes via imperfect and/or perfect complementary base pairing between a miRNA guide strand and the 3' UTR region of the target mRNA, which causes translational repression or mRNA degradation ^{15,17}. In a pathological state such as cancer, the expression levels of multiple miRNAs are aberrantly up- or downregulated, resulting in an imbalance of cellular pathways. In **chapter 2**, we describe dysregulated miRNAs in cells of the tumor stroma and their functions in the regulation of the tumor microenvironment. Additionally, we describe how dysregulated miRNA levels can be controlled by administering miRNA inhibitors (anti-miRNA oligonucleotides) or miRNA agonists (miRNA mimics). Finally, we summarize various miRNA delivery approaches that have been or can potentially be applied to deliver miRNA as therapeutics into stromal cells.

In **chapter 3**, we present a novel peptide based miRNA delivery system to deliver anti-miRNA oligonucleotides (AMOs) to hPSCs. A dimeric form of an arginine- rich cell penetrating peptide (CPP) was synthesized and used for preparing self-assembling nanocomplexes (NCs) with AMOs, via charge-charge interactions. Intriguingly, these NCs showed significantly higher uptake by hPSCs compared with the NCs prepared with the monomeric form. Although branching or multivalent approaches may enhance CPP-mediated uptake, our dimeric CPP-based NCs were preferentially internalized in hPSCs compared with normal fibroblasts or pancreatic tumor cells. This led us to examine different uptake mechanisms, by which hPSCs take up these NCs, showing their uptake via receptor-mediated endocytosis. In our lab, we have previously identified miRNA-199a as a novel therapeutic target in CAFs of PDAC patients and demonstrated that miRNA-199a was induced in patient-derived CAFs as well as activated hPSCs ¹⁸. Furthermore miRNA-199a was shown to regulate the activation of hPSCs and hPSC-induced tumor promoting effects on tumor cells ¹⁸. Therefore, we utilized our NCs for specific delivery of an AMO against miR-199a to hPSCs in 2D cell cultures and 3D-heterospheroids. Interestingly,

delivery of anti-miR-199a into hPSCs inhibited their TGF- β -mediated activation and hPSC-mediated paracrine effects on tumor cell growth.

In **chapter 4**, we looked into inflammation resolving bioactive lipids as potential therapeutics to dampen the function of CAFs and thereby reprogram the tumor stroma. We identified lipoxin A4 (LXA4), a highly potent endogenous lipid, known for its anti-inflammatory and anti-fibrotic function as a potential therapeutic to inhibit the tumor promoting function of activated hPSC¹⁹⁻²¹. We showed that The LXA4-specific surface receptor FPR2/ALX was upregulated in TGF β activated hPSCs. Treatment of hPSCs with LXA4 inhibited TGF- β mediated induction of hPSC activation markers (e.g. α SMA, collagen, PDGFR β), migration and morphological changes in hPSC and therefore the activation of hPSCs into CAF-like myofibroblasts *in vitro*. Additionally, LXA4 inhibited hPSC-induced tumor-promoting paracrine effects, including tumor cell proliferation and migration. In our novel 3D-heterospheroidal *in vitro* tumor model, consisting of hPSCs and Panc-1 tumor cells, LXA4 could inhibit the activation of hPSCs within the 3D microenvironment and reduced spheroid growth and ECM deposition within the spheroids. Finally, we showed the therapeutic efficacy of LXA4 *in vivo* in an hPSC/Panc-1 co-injection tumor model, in which LXA4 reduced tumor growth and ECM-deposition. This study highlights LXA4 as a potential biological to be developed for the treatment of pancreatic cancer.

Next to miRNA and bioactive lipids, we investigated integrins as therapeutic and drug delivery targets, since integrin signaling is critical for cell activation (differentiation), an integration of signaling networks with a role in tumor progression, invasion and metastasis^{22,23}. In **Chapter 5** we provide a general introduction of integrins and integrin-mediated signaling, an overview of integrin expression in fibrosis-related cell types, and the interaction between integrins and growth factor receptors. The next section describes the role of integrins in wound healing, fibrosis and tumor stroma. The final two sections are focused on the novel therapies based on the inhibition of integrin receptors, including clinical developments, and drug delivery strategies to target integrins. We find implications for various novel therapeutic and drug targeting targets in studies which revealed the biological function of various integrins within fibrosis related disease. However, we conclude that studies exploiting the expression of a specific integrin subunit and its cellular localization within pathological tissues and other healthy organs will be crucial to determine novel integrin-based therapeutic targets. Moreover, the design of highly integrin subunit specific

drugs will decrease the risk of off-target effects due to an increased specificity of these molecules. A clear understanding of integrin subunit – growth factor receptor interactions is an additional major aspects to identify integrin subunits which can be utilized as therapeutic targets.

Chapter 6 introduces the collagen 1 binding transmembrane receptor ITGA11 as a significant regulator of the phenotype and function of CAFs. ITGA11 was shown to be highly induced in the stroma of human PDAC and co-localized with the major CAF marker, α -SMA, in PDAC samples. Strikingly, the knockdown of ITGA11 in hPSCs inhibited their TGF- β -mediated activation, migration and contractility. A gene profiler array for ECM and adhesion molecules and a cytokine array, revealed that many of these factors were downregulated in hPSCs after ITGA11 knockdown or significantly less induced in activated hPSCs with shITGA11 knockdown. Moreover, we show that the knockdown of ITGA11 inhibits hPSCs mediated paracrine effects on Panc-1 tumor cell migration and invasion. In conclusion, ITGA11 was found to be highly and specifically expressed in the tumor stroma of PDAC and this study presents ITGA11 as a crucial target in maintaining the phenotype, activation ability and function of hPSCs. Therefore ITGA11 is expected to have a strong impact on the regulation of hPSC-induced desmoplasia in PDAC.

Activated hPSCs also known as CAFs, significantly contribute to the development of the dense tumor stroma, which prevents drug penetration and thereby causes drug resistance in PDAC. Therefore, drug delivery to pancreatic tumors by targeting CAFs, presents a promising approach to specifically delivery therapeutics to CAFs, thereby modulate the tumor stroma and enhance drug penetration into the tumor. However, no CAF-specific targets have been previously reported ²⁴. In **chapter 7** we have identified the integrin subunit ITGA11, to be specifically expressed on CAFs in the tumor stroma of PDAC. Using phage display we have designed a novel peptide (AXI), specifically binding to ITGA11. We use AXI to deliver liposomes to CAFs *in vitro* and show that AXI binds to ITGA11 on hPSCs, thereby facilitating the uptake of AXI-decorated liposomes into these cells. In vivo, liposomes modified with AXI on their surface showed enhanced accumulation and penetration into the tissue of ITGA11 rich hPSC + Panc-1 tumor bearing mice and increased accumulation of liposomes in ITGA11 positive cells, when compared to liposomes modified with a scrambled version of AXI. In conclusion, this study presents AXI, a novel ITGA11 targeting peptide, which can be utilized for targeted drug delivery to reprogram CAFs and thereby modulate the tumor stroma in pancreatic cancer.

In conclusion, there is an urgent need for novel therapeutic strategies to treat PDAC, since traditional chemotherapies aiming to kill cancer cells are failing to cure PDAC in the clinic. Therefore, this thesis explores strategies to modulate and actively target the tumor stroma in PDAC, which is a rapidly emerging area in oncology. In the context of the tumor stroma we focused on hPSCs, which significantly contribute to tumor progression in PDAC. We show the therapeutic potential of anti-miR-199a oligonucleotides (*in vitro*) and LXA4 (*in vitro* and *in vivo*) to reprogram activated hPSCs and thereby their tumor promoting effects. Additionally, we introduce a novel system to deliver anti-miRNA into hPSCs. Next, we identified ITGA11 as a novel therapeutic target specifically expressed on CAFs in PDAC, which controls the phenotype and function of these cells. We introduce ITGA11 as a high potential target for drug delivery, due to its specific expression in the tumor stroma, which minimizes the risk of off-target effects. Finally, we designed an ITGA11 targeting peptide which showed increased accumulation and penetration depth and the ability to localize in ITGA11-expressing CAFs *in vivo*. For future studies it is highly recommended to combine the CAF-reprogramming therapeutics and drug targeting strategies discovered in this thesis and investigate their effect as combination treatment with chemotherapeutics.

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Appendix A - Nederlandse Samenvatting

Pancreatic ductal adenocarcinoma (PDAC) is het type kanker met de slechtste prognose met een 1-jarig overlevingspercentage van 20% en een 5-jarig overlevingspercentage van slechts 8%. Chirurgische resectie is alleen bij een klein percentage van patiënten mogelijk en daardoor maakt dit radio- of chemotherapie de standaard behandelingsmethode. Er is veel onderzoek uitgevoerd naar het vinden van effectieve behandelmethoden voor PDAC. Tot nu toe zijn er echter alleen marginale verbeteringen in het overlevingspercentage bereikt. Eén van de hoofdoorzaken van het falen van de behandeling van PDAC is de overvloedige desmoplasie in PDAC, ook wel tumor stroma of tumor micro-omgeving genoemd, die de PDAC progressie, invasie en de vorming van metastasen ondersteunt.

Veel voorkomende componenten van het tumor stroma zijn kanker-geassocieerde fibroblasten (CAFs), tumor-geassocieerde macrofagen (TAMs), pericyten, endotheelcellen, infiltrerende immuuncellen en eiwitten van de extracellulaire matrix (ECM) (b.v. collageen). Eén van de meest voorkomende celtypen uit de pancreas tumor stroma zijn CAFs. De voorlopers van CAFs zijn human pancreatic stellate cells (hPSCs). ‘Quiescent’ hPSCs staan bekend om hun vermogen om vitamine A op te slaan, zijn in kleine hoeveelheden in het bindweefsel van de pancreas te vinden en produceren alleen kleine hoeveelheden van ECM eiwitten. Tijdens PDAC progressie, worden ‘quiescent’ hPSCs, door weefsel schade en groeifactoren zoals TGF- β 1, in CAF-achtige myofibroblasten geactiveerd. Als gevolg hiervan verliezen zij de capaciteit om lipiden op te slaan en starten met het produceren van grote hoeveelheden ECM componenten, wat tot desmoplasie leidt. Desmoplasie, een fibrotisch stromaal weefsel, werkt als een fysieke barrière en voorkomt de accumulatie van medicijnen in de tumor. Daarnaast produceren geactiveerde hPSCs verschillende cytokines en groeifactoren, die tumorcellen stimuleren en hierdoor tumorgroei, invasie, metastase en uiteindelijk tumorprogressie faciliteren. Daarom is modulatie van het tumor stroma door het remmen van de functie van geactiveerde hPSCs een innovatieve strategie om de therapie van PDAC te verbeteren.

In dit proefschrift ligt de focus op de identificatie van nieuwe therapeutische ‘targets’, zoals microRNA (miRNA), integrine receptoren en bioactieve lipiden om geactiveerde hPSCs in hun ‘quiescent’ fenotype te reprogrammeren en hierdoor de tumor stroma van PDAC te moduleren. Bovendien hebben wij een

nieuw systeem voor de afgifte van miRNA aan hPSCs en een integrine alpha 11 specifieke peptide voor de doelgerichte afgifte van medicijnen aan geactiveerde hPSCs in het stroma van PDAC ontwikkeld.

In **hoofdstuk 2** introduceren wij het concept van miRNA ontregeling in de tumor stroma, welke een hoofdrol in de ontwikkeling en de progressie van kanker speelt en verschillende signaleringsroutes in het tumor stroma reguleert. Hierdoor zijn anti-miRNAs een potentiële klasse van medicijnen. In dit hoofdstuk beschrijven we ontregelde miRNAs in cellen van het tumor stroma en discussiëren we strategieën voor de doelgerichte afgifte van anti-miRNAs aan deze celtypes.

MicroRNAs zijn op zichzelf niet in staat om met de celmembraan te associëren en worden daarom ook niet door een cel opgenomen. In **hoofdstuk 3** stellen wij een nieuw peptide gebaseerd miRNA afgifte systeem voor om anti-miRNA oligonucleotiden (AMOs) aan hPSCs af te geven. Hiervoor hebben wij nanocomplexen (NCs) met AMOs en een door ons ontworpen cel-penetrerend peptide (CPP) gevormd. Deze NCs vertonen significant meer opname in hPSCs indien vergeleken met alvleesklier tumor cellen en normale fibroblasten. Door de afgifte van anti-miR-199a met dit systeem werd de differentiatie van hPSCs in CAFs, hPSC-induceerde tumorcel groei, de groei van tumor sferoïden bestaande uit hPSCs en tumor cellen en hPSC-gebaseerde tumorcel groei geremd.

In **hoofdstuk 4** hebben wij de ontstekingsremmende bioactieve lipide lipoxin A4 (LXA4) als potentieel potentiële medicijn voor het remmen van de functie van CAFs getest. Behandeling van hPSCs met LXA4 remt hun activatie richting CAFs, gezien aan de verminderde expressie van activatiemarkers, de migratie potentiaal en morfologische veranderingen van hPSCs. Bovendien remt LXA4 tumor-bevorderende effecten van hPSCs, zoals tumorcel groei en migratie. In tumor sferoïden bestaande uit hPSCs en tumorcellen was LXA4 in staat om de activatie van hPSCs in de sferoïden evenals de groei van en ECM depositie in de sferoïden te remmen. Uiteindelijk laten wij de therapeutische werking van LXA4 in een hPSC/tumor cel co-injectie model *in vivo* zien, wat tot verminderde tumorgroei en ECM-depositie in de tumoren leidt. Deze studie accentueert LXA4 als een potentiële ‘biological’ voor de behandeling van PDAC.

Naast miRNA en bioactieve lipides hebben wij integrine als therapeutische en ‘drug delivery targets’ onderzocht, omdat integrine signalering kritisch is voor

celactivatie, differentiatie en de integratie van cel signaleringsroutes met een rol in tumor progressie, invasie en metastase. In **hoofdstuk 5** geven wij een algemene introductie over integrineexpressie in fibrose-gerelateerde celtypes en de interactie tussen integrines en groeifactor receptoren. De volgende sectie beschrijft de rol van integrines in wond genezing, fibrose en de tumor stroma. De laatste twee secties zijn gericht op nieuwe integrine-remmende therapieën, inclusief klinische ontwikkelingen en systemen voor de doelgerichte afgifte van medicijnen aan integrines. Wij concluderen dat de ontwikkeling van integrine-subunit specifieke medicijnen het risico van off-target effecten zou minimaliseren. Een duidelijk begrip van integrine subunit – groeifactor receptor interacties is een ander belangrijk aspect om integrine-subunits te identificeren, welke als therapeutische targets benut kunnen worden.

In **hoofdstuk 6** stellen wij de transmembraanreceptor integrin alpha 11 (ITGA11) als regulator van de fenotype van CAFs voor. ITGA11 heeft een hoge expressie in het stroma van PDAC en is gelokaliseerd op CAFs. Het uitschakelen van ITGA11 in hPSCs remt hun activatie, migratie en contractie vermogen. Veel ECM- en celadhesiemoleculen komen in hPSCs met uitgeschakeld ITGA11 minder tot expressie of zijn minder geïnduceerd in geactiveerde hPSCs met uitgeschakeld ITGA11. Bovendien laten wij zien dat het uitschakelen van ITGA11 hPSC-geïnduceerd effecten op tumorcellen, zoals migratie en invasie remt. Concluderend hebben wij gevonden dat ITGA11 verhoogd en specifiek in de stroma van PDAC tot expressie komt en een belangrijke target is in het behoud van het fenotype, activatiepotentiaal en functie van hPSCs. Hierdoor wordt verwacht dat ITGA11 een sterke rol in de regulatie van hPSC-geïnduceerde desmoplasie in PDAC speelt.

Geactiveerde hPSCs, ook bekend als CAFs, dragen significant aan de ontwikkeling van de tumor stroma in PDAC bij, wat de penetratie van medicijnen in het stroma vermindert. Daarom is de doelgericht medicijnafgifte aan CAFs in PDAC tumoren een veelbelovende strategie om de tumor stroma te moduleren en de penetratie van medicijnen in de stroma te verhogen. Er zijn echter nog geen CAF-specifieke targets bekend.

In **hoofdstuk 7** hebben wij ITGA11 als specifieke CAF-marker in de tumor stroma van PDAC geïdentificeerd. Met behulp van de ‘phage display’ techniek hebben wij een nieuw peptide (AXI), met specifieke bindingsaffiniteit voor ITGA11 ontwikkeld. Wij hebben AXI gebruikt om liposomen via ITGA11 aan CAFs af te geven. Hierbij wordt de opname van AXI-gemodificeerde

liposomen door ITGA11 faciliteert. *In vivo* laten AXI-gemodificeerde liposomen, vergeleken met liposomen gemodificeerd met een ‘scrambled’ versie van AXI (sAXI), een verhoogde accumulatie en penetratie in tumoren in muizen en ITGA11-positieve cellen zien. Concluderend hebben wij in deze studie een nieuwe ITGA11 ‘targeting’ peptide ontwikkeld, welke voor de doelgerichte afgifte van medicijnen voor het reprogrammeren van CAFs en het moduleren van de tumor stroma in PDAC gebruikt kan worden.

Samenvattend is er een dringende behoefte voor nieuwe therapeutische strategieën voor de behandeling van PDAC, omdat traditioneel gebruikte chemotherapeutische therapieën falen om patiënten met PDAC te genezen. Daarom worden in dit proefschrift strategieën voor de modulatie van en doelgerichte medicijnafgifte aan de tumor stroma in PDAC onderzocht. In toekomstige studies wordt aanbevolen de CAF-reprogrammerende medicijnen en strategieën voor doelgerichte medicijnafgifte, ontdekt in dit proefschrift, te combineren en hun effect als combinatie behandeling met chemotherapie te evalueren.

Appendix B

List of Publications

Manuscript in preparation:

“A novel peptide against integrin $\alpha 11$ to target nanoparticles to cancer-associated fibroblasts.”

Schnittert J *et al.*

“Integrin $\alpha 11$ regulates differentiation of pancreatic stellate cells in pancreatic cancer.”

Schnittert J *et al.*

“Pancreatic stellate cells: Are they the stars of pancreatic cancer?”

Schnittert J *et al.*

Submitted

"Targeting integrin $\alpha 5$ receptor in pancreatic stellate cells to diminish tumor-promoting effects in pancreatic cancer"

Kuninty PR, Bansal R, De Geus SWL, **Schnittert J**, van Baarlen J, Storm G, Bijlsma MF, van Laarhoven HW, Kuppen PJK, Vahrmeijer AL, Ostman A, Sier CFM, Prakash J

Submitted to Nature Communications

Published:

“Integrins in wound healing, fibrosis and tumor stroma: High potential targets for therapeutics and drug delivery.”

Schnittert J, Bansal R, Storm G, Prakash J.

Adv Drug Deliv Rev. 2018 Feb 4. pii: S0169-409X(18)30028-0. doi: 10.1016/j.addr.2018.01.020.

PMID: 29414674

“Reprogramming tumor stroma using an endogenous lipid lipoxin A4 to treat pancreatic cancer.”

Schnittert J, Heinrich MA, Kuninty PR, Storm G, Prakash J.

Cancer Lett. 2018 Feb 2. pii: S0304-3835(18)30105-8. doi: 10.1016/j.canlet.2018.01.072.

PMID: 29408203

“Anti-microRNA targeting using peptide-based nanocomplexes to inhibit differentiation of human pancreatic stellate cells.”

Schnittert J, Kuninty PR, Bystry TF, Brock R, Storm G, Prakash J.
Nanomedicine (Lond). 2017 May 19. doi: 10.2217/nnm-2017-0054.
PMID: 28524768

“Stroma-regulated HMGA2 is an independent prognostic marker in PDAC and AAC.”

Strell C, Norberg KJ, Mezheyeuski A, **Schnittert J**, Kuninty PR, Moro CF, Paulsson J, Schultz NA, Calatayud D, Löhr JM, Frings O, Verbeke CS, Heuchel RL, Prakash J, Johansen JS, Östman A.
Br J Cancer. 2017 Jun 27;117(1):65-77. doi: 10.1038/bjc.2017.140. Epub 2017 May 18.
PMID: 28524160

“MicroRNA Targeting to Modulate Tumor Microenvironment.”

Kuninty PR, **Schnittert J**, Storm G, Prakash J.
Front Oncol. 2016 Jan 19;6:3. doi: 10.3389/fonc.2016.00003. eCollection 2016. Review.
PMID: 26835418

Scientific Awards

Oral Free Paper Price

United European Gastroenterology (UEG) Week, October 2017

Best Poster / Small Speech Award

Annual meeting of CLINAM (European Foundation for Clinical Nanomedicine), June 2016

Appendix C – Acknowledgements

After almost 3.5 years I am now spending one of the last evening in my office ZH253 and have a very hard time to believe that the time has come to write this important acknowledgment section of my thesis. All this time went unbelievably quick. My plan was to keep it short. That did not work.

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