Pancreatic Stellate Cells promote hapto-migration of Pancreatic Cancer Cells through Collagen I-initiated signaling pathway

Dissertation
Applying for the Doctoral Degree of Medicine (Dr. rer. med)
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- October 2013 -
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Tag der Promotion: 19-12-2013
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<thead>
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<tbody>
<tr>
<td>APS</td>
<td>Ammoniumperoxodisulfate</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Col</td>
<td>collagen</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>FGF-2</td>
<td>fibroblast growth factor-2</td>
</tr>
<tr>
<td>FAs</td>
<td>focal adhesions</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fn</td>
<td>fibronectin</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>PDAC</td>
<td>pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PanIN</td>
<td>pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PSC</td>
<td>pancreatic stellate cell</td>
</tr>
<tr>
<td>PCC</td>
<td>pancreatic cancer cell</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PSC-SN</td>
<td>PSC supernatant</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
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<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-L-lysine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SFM</td>
<td>serum-free medium</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interference RNA</td>
</tr>
<tr>
<td>TNB</td>
<td>Tris-buffered saline containing bovine serum albumin</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor-β1</td>
</tr>
</tbody>
</table>
Pancreatic cancer is one of the 15 most common cancers worldwide, with a gradually increased incidence during the 2000-2008 period (Ferlay et al. 2010; Parkin 2001). Because of its rapid progression, early metastasis, diagnosis at an advanced-stage, and limited responses to chemotherapy and radiotherapy (Bachem et al. 2005), pancreatic cancer shows an exceptionally high mortality rate. It ranks as the 8-9th leading cause of cancer deaths in the world, and as the 4-5th in most developed countries (Raimondi et al. 2009). A better understanding of the molecular biology of pancreatic cancer is critically required for effective therapeutic strategies in the future.

Pancreatic ductal adenocarcinoma (PDAC) accounts for over 90% of pancreatic malignancies (Stathis and Moore 2010). The defining feature as well as the histological hallmark of PDAC is the profound desmoplastic reaction surrounding the tumor tissue (Bardeesy and DePinho 2002; Cubilla and Fitzgerald 1975; Mollenhauer et al. 1987). Several studies (Couvelard et al. 2005; Infante et al. 2007; Mantoni et al. 2008; Watanabe et al. 2003) suggest that the presence of stroma in PDAC correlates with reduced survival of patients. In normal subjects without any clinically apparent pancreatic disease, fibrosis was found to be commonly associated with pancreatic intraepithelial neoplasia (PanIN) (Detlefsen et al. 2005), a precursor to invasive pancreatic cancers (Hruban et al. 2008). Moreover, in PanINs mouse models, desmoplastic reactions were observed around the premalignant lesions (Aguirre et al. 2003), and an increased proliferation of stroma tissue was in company with the advanced neoplastic progression (Bardeesy et al. 2006). So these findings strongly indicate that desmoplastic stroma is a prominent microenvironment responsible for the malignant biology of pancreatic cancer.

The desmoplasia in pancreatic cancer is composed of copious amounts of extracellular matrix (ECM) and various types of non-malignant cells, such as mesenchymal cells, vascular cells, and immune cells. The ECM proteins that have been characterized in PDAC are collagen (type I, III, IV), fibronectin, laminin, and vitronectin (Gress et al. 1995; Linder et al. 2001; Ryschich et al. 2009; Shimoyama et al. 1995), with collagen type I and fibronectin to be the most abundant. In a quantitative analysis (Imamura et al. 1995), there was a 3-fold increase of the collagen content in PDAC compared with the normal pancreas. The major cell type responsible for the matrix production has been identified as pancreatic stellate cell (PSC) (Apte et al. 2004; Bachem et al. 2005), which is kind of mesenchymal
resident cell. In normal pancreas, PSCs localize in the interlobular and periacinar space, showing a quiescent phenotype characterized by the numerous perinuclear fat-droplets, low mitotic index and low synthesis capacity of ECM (Apte et al. 1998; Bachem et al. 1998). During pancreatic injury or in vitro culture, PSCs are activated and transformed to a myofibroblast-like phenotype. Activated PSCs, which are present in the fibrotic area of injured pancreas in vivo, lose the retinoid containing droplets, express \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), have a high mitotic index, and produce excessive amounts of ECM components collagen type I and III and fibronectin.

In the light of the contribution of PSCs to desmoplasia and the spatial proximity to cancer cells, increasing attention is being paid to the interaction between PSCs and cancer cells in the progression of PDAC. Our previous work shows that pancreatic cancer cells (PCCs) stimulate the motility (Bachem et al. 2008), proliferation and matrix synthesis (Bachem et al. 2005) of PSCs in a paracrine way, via soluble factors including transforming growth factor-\( \beta 1 \) (TGF-\( \beta 1 \)), fibroblast growth factor-2 (FGF-2), and platelet-derived growth factor (PDGF). The result of this stimulation is the increased deposition of fibrotic tissue surrounding tumors. Vice versa, activated PSCs accelerate proliferation of PCCs in vitro (Bachem et al. 2008), induce tumor invasion in CAM assay (Schneiderhan et al. 2007), and support subcutaneous tumor growth in nude mouse models (Bachem et al. 2005). In line with our findings, other research groups (Hwang et al. 2008; Vonlaufen et al. 2008) have also shown that in vitro PSC supernatant stimulates the migration, invasion and colony formation of PCC, while in vivo co-injection of PCCs with PSCs into orthotopic murine models results in increased primary tumor incidence, size, as well as distant metastasis. Xu et al. (Xu et al. 2010) even suggest that PSCs are able to accompany cancer cells to metastatic sites and stimulate angiogenesis. The above findings demonstrate a reciprocal interaction: that PSCs are recruited and activated by PCCs, which in turn produce a beneficial environment to promote local tumor progress and metastatic expansion. Nevertheless, the precise biological mechanisms involved in PSC-induced malignancy, in particular in the induction of metastasis, are still elusive.

Cancer metastasis consists of sequential and interlinked steps, among which cell migration is an essential component. Different modes of cell locomotion are initiated in responses to different extracellular cues, including soluble or surface-attached chemicals, light intensity, electrostatic potential and matrix rigidity (Petrie et al. 2009). Chemotaxis, representing directed cell migration towards gradients of soluble chemokines, is important for efficient
tumor metastasis and has been studied extensively in vitro and in vivo (Roussos et al. 2011). Haptotaxis, firstly put forward by Cater (Carter 1967), demonstrates the capacity of motile cells to direct their movement along substrate-bound chemicals (ECM proteins). In vivo, both two-dimensional surfaces (inner vessel walls, peritoneum, pleura, etc.) and three-dimensional interstitium composed of fibril ECM components may serve as “substrates” for cells (Friedl and Weigelin 2008). On the other side, cancer cells constitutively expressing adhesion receptors (Herrera-Gayol and Jothy 1999; Lyons and Jones 2007) bind to ECM, and the formed adhesive structures not only provide structural support, but also deliver important signals for cell survival (Hehlgans et al. 2007), migration (Palmer et al. 2011), and invasion (Gimona and Buccione 2006).

Boyden chamber, originally introduced by Boyden in 1960s, is a useful tool for the quantitative analysis of different cell locomotion. Basically, 4 forms of cell locomotion could be characterized in this assay. Chemotaxis is induced by adding soluble chemokines to the lower chamber, chemokinesis by adding to both upper and lower chambers, haptotaxis by coating the underside of membrane with substratum-bound factors while haptokinesis is by coating both sides of the membrane (Douglas-Escobar et al. 2012; Klominek et al. 1993). Moreover, cell trans-migration in Boyden chamber mimics the in vivo process of cell migration, which consists of several cell biological features, including adhesion, motility, directionality and deformation.

In this study, the modified Boyden chamber assay was applied as an in vitro model to investigate the effect of PSCs on trans-migration of PCCs. Considering that PSCs produce excessive amounts of ECM proteins (fibronectin, collagen I, III) as well as multiple soluble chemokines, each type of cell locomotion described above might be induced in PCCs. To further characterize the cellular and molecular mechanisms underlying PSC-stimulated PCC trans-migration will shed light on the understanding of PSC-promoted progress of pancreatic cancer.

**Questions and Aims —**

In this study, we aim to answer the following questions:

- Do PSCs stimulate the trans-migration of PCCs in the modified Boyden chamber?
- Which biological steps involved in PCC trans-migration (e.g. adhesion, motility, directional migration) is/are affected by PSCs?
- Which factor produced by PSCs is mainly responsible for PSC-induced PCC trans-migration?
• Which intracellular signaling is initiated upon PSC-induced PCC trans-migration?

To answer these questions, human PSCs were isolated from tissues of pancreatic cancer or chronic pancreatitis obtained through pancreas surgery. Conditioned medium was collected from sub-confluent PSCs. The effect of PSC supernatant (PSC-SN), as well as adhesive molecules, on the trans-migration, adhesion and motility of PCCs (Panc1, UlaPaCa) was investigated by modified Boyden chamber assay, adhesion assay and single cell tracking assay, respectively. Organization of cytoskeleton and formation of focal adhesions (FAs) were examined by fluorescence microscopy of F-actin, paxillin and phospho-focal adhesion kinase (FAK, Tyr397). Integrin expression and FAK activity were assessed by Western blot. Anti-integrin α2/β1 antibodies and FAK inhibition by either PF-573228 or siRNAs were used to demonstrate the role of collagen I and the involved signaling pathway.
2. MATERIALS AND METHODS

2.1 Materials
2.1.1 Chemical reagents

Acetone Fluka BioChemika, Buchs, Switzerland
AEBSF, hydrochloride Calbiochem, EMD Millipore, San Diego, CA, USA
Ammoniumperoxodisulfate (APS) Merck, Darmstadt, Germany
Aprotinin Merck, Darmstadt, Germany
Bromophenol blue Merck, Darmstadt, Germany
Dimethylsulfoxid (DMSO) Sigma, Steinheim, Germany
Dulbecco’s modified Eagle medium Biochrom AG, Berlin, Germany
(DMEM)/Ham’s F-12 medium (1:1)
Dulbecco PBS, wo Ca/Mg Biochrom, Berlin, Germany
Dulbecco’s phosphate buffered saline Life technologies, Paisley, UK
(PBS) (1×, wo Ca/Mg)
EDTA Applichem, Darmstadt, Germany
Formaldehyde solution 3.5-3.7% Otto Fischar, Saarbrücken, Germany
Glycine Sigma, St. Louis, MO, USA
Glycerol Merck, Darmstadt, Germany
Hoechst 33258 Sigma, St. Louis, MO, USA
Hydrochloric acid VWR, Haasrode, Belgium
Leupeptin (hemisulfate salt) Sigma, Steinheim, Germany
Methanol Sigma, St. Louis, MO, USA
Paraformaldehyde (PFA) Fluka BioChemika, Buchs, Switzerland
Pepstatin A Fluka BioChemika, Buchs, Switzerland
20% SDS Solution SERVA, Kalsruhe, Germany
### MATERIALS AND METHODS

(Sodium dodecyl sulfate. Na)

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baysilone–Paste hochviskos</td>
<td>GE. Bayer Silicones, Leverkusen, Germany</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>VWR, Haasrode, Belgium</td>
</tr>
<tr>
<td>Sodium orthovanadate (Na&lt;sub&gt;3&lt;/sub&gt;VO&lt;sub&gt;4&lt;/sub&gt;)</td>
<td>Sigma, Steinheim, Germany</td>
</tr>
<tr>
<td>di-Sodium dihydrogen pyrophosphate (Sodium pyrophosphate)</td>
<td>Fluka BioChemika, Buchs, Switzerland</td>
</tr>
<tr>
<td>Sodium fluoride (NaF)</td>
<td>Fluka BioChemika, Buchs, Switzerland</td>
</tr>
<tr>
<td>Sterile water (ddH&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>Fresenius Kabi, Bad Homburg, Germany</td>
</tr>
<tr>
<td>Tetramethylethlenediamine (TEMED)</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Tris</td>
<td>USB corporation, Ohio, USA</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Trypsin/EDTA solution</td>
<td>PAA, Pasching, Austria</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma, Steinheim, Germany</td>
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#### 2.1.2 Biological reagents

<table>
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<tr>
<td>Alexa Fluor 568 conjugated-streptavidin</td>
<td>MolecularProbes, Eugene, OR, USA</td>
</tr>
<tr>
<td>Alexa Fluor 488-Phalloidin</td>
<td>MolecularProbes, Eugene, OR, USA</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Boehringer, Mannheim, Germany</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>PAA Laboratories GmbH, Austria</td>
</tr>
<tr>
<td>Collagen AP1</td>
<td>Matrix BioScience, Morlenbach, Germany</td>
</tr>
<tr>
<td>Fetal bovine serum (FBS)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>EMP Genetech, Ingolstadt, Germany</td>
</tr>
<tr>
<td>H-Arg-Gly-Asp-OH peptide (RGD)</td>
<td>Bachem AG, Bubendorf, Switzerland</td>
</tr>
<tr>
<td>Laminin</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>L-glutamine with penicillin/streptomycin</td>
<td>PAA, Pasching, Austria</td>
</tr>
<tr>
<td>TSA™ Biotin System reagent NEN</td>
<td>Life Science Products, Boston, USA</td>
</tr>
<tr>
<td>Poly-L-lysine hydrobromide</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
</tbody>
</table>
2.1.3 Antibodies

- Allexa Fluor 594 F (ab’)2 fragment of rabbit anti-mouse IgG (H+C) 
  Molecular Probes, Eugene, OR, USA
- Allexa Fluor 488 F (ab’)2 fragment of rabbit anti-mouse IgG (H+C) 
  Molecular Probes, Eugene, OR, USA
- Alexa Fluor568 conjugated-Streptavidin 
  Molecular Probes, Eugene, OR, USA
- Mouse anti-human integrin α1 I domain, clone FB12, azide free 
  Millipore, Temecula, CA, USA
- Mouse anti-human integrin α2, clone P1E6, azide free 
  Millipore, Temecula, CA, USA
- Mouse anti-human integrin β1, clone P4C10, azide free 
  Millipore, Temecula, CA, USA
- Mouse anti-human VLA-2 (α2β1) integrin, clone BHA2.1, azide free 
  Millipore, Temecula, CA, USA
- Mouse anti-human desmin 
  DAKO, Glostrup, Denmark
- Purified mouse anti-human FAK (Y397), clone 14/FAK (Y397) 
  BD Biosciences, Bedford, MA, USA
- Mouse anti-paxillin, clone 5H11 
  Millipore, Temecula, CA, USA
- Mouse anti-human α-SMA 
  DAKO, Glostrup, Denmark
- Mouse anti-phosphor-tyrosine (P-Tyr-100) 
  Cell signaling, Danvers, MA, USA
- Mouse anti-vimentin, clone V9 
  DAKO, Glostrup, Denmark
- Rabbit anti-human integrin α2 (CD49b) 
  Millipore, Temecula, CA, USA
- Rabbit anti-FAK polyclonal antibody 
  Millipore, Temecula, CA, USA
- Rabbit anti-mouse HRP 
  DAKO, Glostrup, Denmark
- Swine anti-rabbit HRP 
  DAKO, Glostrup, Denmark
- Purified mouse anti-β tubulin, clone D66 
  Sigma, St. Louis, MO, USA
2.1.4 Inhibitor of signaling pathway

**Focal adhesion kinase inhibitor II** (Calbiochem, EMD Millipore, CA, USA)

A cell-permeable pyrimidinyldiamino compound that acts as a selective, ATP-competitive, and potent FAK inhibitor ($IC_{50} = 4$ nM). PF-573228 effectively inhibits cellular FAK Tyr397 phosphorylation in various human, rat, and canine cell lines ($IC_{50} = \sim 30$-500 nM during a 1 h incubation period).

Stock solution: 1 mM in DMSO, stored at –20 °C.

2.1.5 small interference RNA (Qiagen, Germantown, MD, USA)

**Hs_PTK2_5**

A small interference RNA target human FAK.

Target sequence: 5’-CCGGTCGAATGATAAGGTGTA-3’

Sense strand: 5’-CCUGGGUACUGGUAUGGAATT-3’

Antisense strand: 5’-UUCCAUACCAGUACCCAGGTG-3’

**Hs_PTK2_10**

A small interference RNA target human FAK.

Target sequence: 5’-CCGGTCGAATGATAAGGTGTA-3’

Sense strand: 5’-GGUCGAAUGAUAAGGUGUATT-3’

Antisense strand: 5’-UACACCUUAUCAUUGCGACCGG-3’

**AllStars Neg. siRNA AF 488**

This is a non-silencing siRNA with Alexa Fluor 488 modification which has no homology to any known mammalian gene. It is used as a negative control for non-specific silencing events.

**HiPerFect Transfection Reagent**

A blend of cationic and neutral lipids enables effective siRNA uptake and efficient release of siRNA inside cells.

2.1.6 Assay kits

2.1.6.1 **DC protein assay kit** (Bio-Rad Laboratory, Hercules, CA, USA)

Reagent A 250 ml, an alkaline copper tartrate solution

Reagent B 1L, a dilute Folin Reagent

Reagent S 5 ml

2.1.6.2 **SuperSignal® West Pico Chemiluminescent substrate kit** (Thermo Scientific, Rockford, IL, USA)

Luminol stable peroxide solution 500 ml
### MATERIALS AND METHODS

Luminol Enhancer solution 500 ml

#### 2.1.7 Solutions

##### 2.1.7.1 Solutions for fluorescence staining

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNB buffer</td>
<td>0.1 M Tris HCL, 0.15 M NaCl, 0.5% Albumin, pH 7.5</td>
</tr>
<tr>
<td>0.05% PBST buffer</td>
<td>0.05% Tween 20 in 1 M PBS</td>
</tr>
</tbody>
</table>

##### 2.1.7.2 Solutions for Western blot

#### 2.1.7.2.1 Ready solutions

<table>
<thead>
<tr>
<th>Solution</th>
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</tr>
</thead>
<tbody>
<tr>
<td>10× SDS-PAGE</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Gel A (29% Methylenebisacrylamide)</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Gel B (30% Acrylamide)</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Stripping buffer (Restore™ Western Blot)</td>
<td>Thermo Scientific, Rockford, IL, USA</td>
</tr>
<tr>
<td>Precision Plus Protein™ Standards</td>
<td>Bio-Rad Laboratories, Hercules, CA, USA</td>
</tr>
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#### 2.1.7.2.1 Prepared solutions

<table>
<thead>
<tr>
<th>Solution</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer (for lysing cells to detect phosphorylated proteins)</td>
<td>10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100; 1 mM Na&lt;sub&gt;3&lt;/sub&gt;VO&lt;sub&gt;4&lt;/sub&gt;, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 25 mg/ml AEBSF (freshly added)</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>100 mg APS in 1 ml ddH2O</td>
</tr>
<tr>
<td>0.75 M Tris HCl</td>
<td>90 g Tris in 1 L ddH2O, pH 8.8</td>
</tr>
<tr>
<td>1.5 M Tris HCl</td>
<td>181.7 g Tris in 1 L ddH2O, pH 6.8</td>
</tr>
<tr>
<td>0.625 M Tris HCl</td>
<td>37.84 g Tris in 500 ml ddH2O, pH 6.8</td>
</tr>
<tr>
<td>AEBSF solution</td>
<td>50 mg AEBSF in 2 ml</td>
</tr>
<tr>
<td>8% Separation Gel (25 ml)</td>
<td>6.4 ml gel A, 4 ml gel B, 124 µl 20% SDS, 14.45 ml 0.75 M Tris/HCl (pH 8.8), 200 µl</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

APS, 15 µl TEMED

6% Separation Gel (25 ml)
4.87 ml gel A, 1.95 ml gel B, 125 µl 20% SDS, 6.25 ml 1.5 M Tris/HCl (pH 8.8), 11.555 µl ddH₂O, 250 µl APS, 25 µl TEMED

Stacking gel (10 ml)
933 µl gel A, 736 µl gel B, 50 µl 20% SDS, 1.25 ml 0.625 M Tris/HCl (pH 6.8), 7.03 ml ddH₂O, 100 µl APS, 10 µl TEMED

6× Laemmli sample buffer
156.25 mM Tris/HCl (pH 6.8), 12% SDS (w/v), 0.05% Bromophenol Blue (w/v), 60% Glycerol, 15% β-mercaptoethanol

Running buffer
10× SDS-PAGE buffer in 1L ddH₂O

Transfer buffer
39 mM Glycine, 48 mM Tris/HCl, 0.0375% SDS (w/v), 20% methanol

0.1% PBST buffer (washing buffer)
0.1% Tween 20 in 1 M PBS

Blocking buffer (also used as the antibody dilution buffer)
5 g BSA in 100 ml 0.1% PBST buffer

2.1.8 Equipment

CO₂ incubator
TYP. BB-6220, Heraeus instruments, Germany

Sterile bench
TYP. HS 18/2, Heraeus instruments, Germany

FALCON® cell culture flask (250 ml, 75 cm²)
Becton Dickinson labware, Franklin lakes, NJ, USA

BD FALCON™ polystyrene conical tube (15 ml, 50 ml)
BD Biosciences, Bedford, MA, USA

6/12/24-well cell culture plate
Becton and Dickinson labware, Franklin Lakes, NJ, USA

FALCON® Cell scraper
Becton Dickinson labware, NJ, USA
Koettermann water bath Köttermann GmbH, Wien, Austria

Pipette tips Eppendorf, Hamburg, Germany

Adjustable-volume pipette Eppendorf, Hamburg, Germany

Transwell® with 8.0 µm Pore Polycarbonate Membrane Insert (6.5mm) Corning incorporation, Corning, NY, USA

CryoPure tubes SARSTET AG & CO., Nuembrecht, Germany

NALGENE™ Cryo 1°C Freezing Container Thermo Scientific, Rockford, IL, USA

Liquid nitrogen tank Taylor-Wharton, Theodore, AL, USA

Centrifuge (ROTIXA/RP) Hettich, Tuttlingen, Germany

Centrifuge 5417C Eppendorf, Hamburg, Germany

High speed centrifuge, Biofuge Stratos Heraeus Instruments, Fellbach, Germany

Invert microscope (TELA V AC31) Carl Zeiss, Jena, Germany

Fluorescence microscope (AXIOSKOP) Carl Zeiss, Jena, Germany

Olympus IX81 Motorized Inverted Microscope with F-view II camera Olympus GmbH, Hamburg, Germany

Cell R Imaging Software (Version 1.2) Olympus Biosystems, Planegg, Germany

Digital pH meter pH525, WTW, Weilheim, Germany

Eppendorf Thermomixer 5436 Eppendorf, Hamburg, Germany

Vortex-2TM genie Model G-560E, Scientific Industries, Inc. Bohemia, NY, USA

Platform Shaker (Polymax 1040) LabSource, Heidolph, Germany

DAKO pen S 2002 DAKO, Glostrup, Denmark

MENZEL-GLASER (cut edges frosted end) Thermo Scientific, Schwerte, Germany

Victor 1420 Multilabel Counter Wallac Oy, Turku, Finland

Electrophoresis apparatus Mini protein® 3 cell Bio-RAD, Hercules, CA, USA
2.2 Methods

2.2.1 Cell isolation

Human pancreatic stellate cells (PSCs) were isolated by out-growth method as described previously (Bachem et al. 1998). Pancreatic tissue blocks were obtained during primary surgical resection from patients with pancreatic cancer or chronic pancreatitis. The tumor blocks were minced (0.5-1.0 mm$^3$) using a razor blade, and seeded in 6-well plates (3-5 pieces/well) containing Dulbecco’s modified Eagle medium (DMEM)/ Ham’s F12 supplemented with 20% fetal bovine serum (FBS), L-glutamine plus penicillin/streptomycin (1×) and 1.25 µg/ml amphotericin B. Eighteen hours after seeding, culture medium was changed, and 24 hours later, the tissue blocks were transferred to new culture plates. The PSCs grew out in high number and purity from the tissue blocks 1-3 days later. After reaching confluent, monolayers were trypsinized and passaged 1:3. The whole culture was maintained at 37°C in a humidified atmosphere of 5% CO$_2$.

Cell purity was assessed by morphology (most cells were stellate-like with cytoplasmic extensions; some were spindle shaped), and cytofilament stainings of $\alpha$-SMA (> 95%), vimentin (100%), and desmin (20-40%). PSCs between 3-8 passages were used for the experiments.

2.2.2 Cell culture

2.2.2.1 Pancreatic cancer cell lines
Panc1, MiaPaCa-2 and AsPC-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). UlaPaCa cells were established in our own lab from a peritoneal metastasis of a 71-year-old female patient with PDAC (Haag et al. 2011). Melanoma cell line A375 was the generous gift from Prof. Karin Scharffetter-Kochanek.

### 2.2.2.2 Preparation of cell culture medium

The culture medium used for PSCs and pancreatic cancer cells (PCCs) were same. Complete growth medium is prepared by supplementing DMEM/Ham’s F12 medium with 10% FBS, L-glutamine plus penicillin/streptomycin (1×) and 1.25 µg/ml amphotericin B. In some experiments, cells were suspended or seeded in serum-free medium (SFM) which refers to DMEM/Ham’s F12 medium containing L-glutamine plus penicillin/streptomycin but without FBS.

### 2.2.2.3 Cell culture in culture vessels

PSCs as well as PCCs and A375 were cultured in cell culture flasks (75 cm²) at 37°C in a 5% CO₂ humidified atmosphere. Cells were checked microscopically daily to ensure they were healthy and growing. Every 2-3 days, the growth medium was changed by carefully aspirating out the medium and immediately replacing it with 20 ml fresh pre-warmed growth medium. According to different experimental designs, PCCs were seeded with SFM or growth medium in cell culture plates (6, 12, 24-well plates) or inserts of modified Boyden chamber, or on 1cm² glass coverslips in 6-well plate (3 coverslips/well).

### 2.2.2.4 Subculturing Cells

When PSCs or PCCs were approximately 90% confluent, cells were washed with Dulbecco’s phosphate buffered saline (PBS, w/o Ca²⁺ and Mg²⁺) twice, and 1.5 ml trypsin/EDTA (1×) were added thereafter. The flasks were rotated to cover the entire surface with trypsin and placed in the 37°C incubator for 4-5 minutes. As soon as the cells were detached, 10 ml complete growth medium was added to inactivate the trypsin. The cells were suspended in growth medium, transferred to a 50 ml Falcon tube, and centrifuged at 2000 n/min for 3 minutes. Afterward cells were re-suspended in 1 ml growth medium and a required volume of cell suspension was pipetted into new flasks at certain split ratio (1:3 for PSCs, 1:10 for PCCs).

### 2.2.3 Cryopreservation and reconstitution of cells

The freezing medium is prepared by supplementing DMEM/Ham’s F12 medium with 20% FBS, 10% DMSO, and is stored at 4°C until use. When reaching confluence, cells were
trypsinized, suspended in growth medium, and centrifuged following the procedure during the subculturing. The cell pellet from one flask was re-suspended in 4.8 ml cold freezing medium and dispensed into 3 labeled cryovials, which was further placed into an isopropanol chamber and stored at -80°C overnight. For long term storage, the frozen cells were kept in liquid nitrogen.

For reconstitution, the cryovials containing frozen cells were immediately placed into a 37°C water bath and swirled gently till only a small bit of ice left in the vials. The thawed cells were then transferred to a Falcon tube containing 10 ml pre-warmed growth medium, centrifuged at 2000 n/min for 3 minutes, re-suspended in fresh growth medium, and plated in culture flasks.

2.2.4 Preparation of PSC–conditioned medium
Subconfluent PSCs in 75 cm² flasks were washed twice with PBS. Then 10 ml SFM was added in each flask and conditioned for 48 hours. The serum-free conditioned supernatant from PSCs (PSC-SN) was collected, centrifuged at 2000 n/min, 4°C for 10 minutes, aliquoted into Eppendorf-tubes (1.5 ml or 2 ml) and stored at -20°C. In the following experiments, PSC-SN was diluted with SFM as indicated to evaluate the effect of PSC secretions on the biological behavior of PCCs.

2.2.5 Gene silencing by siRNA
Panc1 cells were seeded with medium containing 10% FBS into 12-well plate at 50% confluence. The cells were allowed to adhere for 1 hour under standard conditions of 37°C and 5% CO₂ humidified atmosphere. For cells in each well, transfection complex was prepared by mixing 4 µl siRNA (10 µM) and 10 µl HiPerFect Transfection Reagent in 86 µl SFM and incubating at room temperature for 20 minutes. Afterward, the 100 µl complex was added drop-wise onto the cells. The final concentration of siRNA was 40 nM. After 48 hours, transfected cells were maintained in fresh medium containing 1% FBS for additional 18 hours before further experimentation.

2.2.6 Cell adhesion assay
24-well plates were filled with SFM or medium containing 10% FBS or 50% PSC-SN (300 µl/well). Cells (4 × 10⁴/well) in SFM were seeded in and allowed to attach for 1 hour. Afterward, non-adherent cells were removed by washing with PBS twice, while adhered cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature and
then stained with Hoechst 33258 (2 µg/ml) for 3 minutes. Nine random fields from each well were taken for digital images and the number of adherent cells was counted at 100 × magnification.

2.2.7 Cell migration assay

Cell migration was assayed by using modified Boyden chambers with 8 µm pore polycarbonate membrane. The schematic illustration of modified Boyden chamber assay was shown in Fig. 1. Lower chambers were filled with 650 µl SFM or medium containing 10% FBS or 50% PSC-SN, the inserts were then placed into these media-containing chambers and pre-incubated at 37°C for at least 20 minutes. Afterward, cells (1 × 10^5/well) in 200 µl SFM were seeded into the inserts, and allowed to trans-migrate for 18 hours at 37°C in 5% CO₂ atmosphere. None-migratory cells on the upper side of the membrane were scraped off with wet cotton swabs. Migrated cells on the underside of the membrane were fixed in 4% PFA and stained with Hoechst 33258 (2 µg/ml) for 3 minutes. Then the membrane was dislodged, rinsed in ddH₂O, and placed on a glass slide. Fluorescence photographs were taken in 7 random fields at 100 × magnification.

Fig. 1. The schematic illustration of modified Boyden chamber assay.

— Media used for stimulation
— SFM for cell seeding
— PCCs
— Microporous membrane
— 4% PFA

— Scratch
— Staining with Hoechst 33258
— Fixation with 4% PFA for 20 min
— Dislodgment
— Evaluation

• PSC-SN
• SFM
• 10% FBS
• PCC suspension
1x10^5 cells/well
Incubation at 37°C for 18 h
Fixation with 4% PFA for 20 min
Scratch
Staining with Hoechst 33258
Dislodgment
Evaluation

Media used for stimulation
SFM for cell seeding
PCCs
Microporous membrane
4% PFA
2.2.8 Single cell tracking assay

12-well plates were filled with SFM, or medium containing 10% FBS or 50% PSC-SN (500 µl/well). Cells (2 x 10⁴/well) were seeded in and allowed to adhere for 1 hour. The culture plate was then placed into a temperature and CO₂-controlled incubator (37°C, 5% CO₂) on the stage of an inverted microscope (Olympus). Time-lapse images were acquired (64 x magnification) every 15-30 minutes for 24 hours under the control of cellIM & cellIR imaging software. In one experiment, cells were seeded in 10% FBS medium for 24 hours, followed by 12-hour starvation in SFM, then stimulated by SFM or 10% FBS or 50% PSC-SN for 3 hours. Time-lapse movie was taken thereafter. For each movie, at least 30 single cells were randomly selected and manually tracked using ImageJ 1.44m (National Institutes of Health, USA). Cell velocity, defined as the length of migration trajectory divided by time, was calculated from the trace of each cell and analyzed in Excel.

2.2.9 Preparation of coated surfaces

In some experiments, cell culture surfaces were coated with poly-L-lysine, collagen I, fibronectin, or laminin.

To coat culture plates, adhesive proteins diluted with PBS as indicated were added into each well and incubated for 1 hour at 37°C. Afterward, the coated surfaces were thoroughly rinsed with PBS and ddH₂O twice respectively, and were air-dried before adding medium or cells. The coated plates, if not used directly, were stored at 4°C for no more than 1 week.

In modified Boyden chamber, the polycarbonate membrane was coated for the investigation of cell haptotaxis or haptokinesis. For haptotaxis, the insert was filled with 100 µl PBS and floated in the lower chamber containing 650 µl poly-L-lysine, collagen I or fibronectin in PBS. After 1 hour incubation at 37°C, the insert was rinsed with PBS and ddH₂O on both sides, air-dried, and placed in another free chamber so that the underside of the membrane with higher concentration of protein faced the lower chamber. For haptokinesis, the insert was filled with the same coating solution as in the lower chamber so that both sides of the membrane were coated with the same concentration of protein, then followed by the other steps as described for haptotaxis.

In one experiment (Fig. 16), the polycarbonate membrane was coated with PSC-SN 1 hour before cell seeding. The insert was filled with 100 µl SFM (for haptotaxis) or 50% PSC-SN
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(for haptokinesis) and floated in the lower chamber containing 650 µl 50% PSC-SN. After
1 hour incubation at 37°C, the insert was rinsed with SFM on both sides and then directly
applied to the experiment.

2.2.10 Western blot analysis

2.2.10.1 Cell lysis and protein extraction

Cells were washed with cold PBS twice, and then cold lysis buffer was added (300 µl/well
for PCCs, 200 µl/well for PSCs). The culture plate was kept on ice with gentle agitation for
10 minutes. Afterward, cells were scrapped off and the lysate was collected to 1.5 ml
Eppendorf-tubes. Insoluble precipitation was discarded after centrifugation at 13000 rpm
for 10 minutes at 4°C. Clarified cell lysate was aliquoted (50 µl/tube) and stored at -20°C
till use. Protein concentration was determined by DC protein assay.

In a time course study, cell lysates were collected at various time points for the detection of
protein phosphorylation. At each time point, suspensions of non-adherent cells were
collected to 2 ml Eppendorf-tubes and placed on ice, while adherent cells were washed
with cold PBS twice and incubated with lysis buffer (200 µl/well) on ice with gentle
agitation for 3 minutes. Lysates of adherent cells were collected to 1.5 ml Eppendorf-tubes
and kept on ice. Suspensions of non-adherent cells were centrifuged at 1000 n/min for 2
minutes at 4°C to discard the supernatant. Pellets of non-adherent cells were suspended in
lysis buffer (50 µl/tube) and collected to the lysates of adherent cells. The total lysates (250
µl/well) were further disrupted by sonication in icy water for 15 seconds for three times.
Afterward, the lysates were clarified, aliquoted and stored as described above.

2.2.10.2 Western blot

Frozen cell lysate was thawed on ice. The lysate (12-30 µg) was mixed with 6× Laemmli
reducing sample buffer and heated at 95°C for 5 minutes. The reduced sample (10-25 µl)
and prestained standard (5 µl) were loaded to and separated on 6% or 8% SDS-PAGE gel
at 130-135 V for 90-120 minutes. Thereafter, the proteins were transferred onto PVDF
membrane with semi-dry electrotransfer system under constant 1.0 mA/cm² of PVDF area
for 90 minutes.

The transferred PVDF membranes were washed in 0.1% PBST for 3 times (5
minutes/time). Nonspecific binding was blocked with 5% BSA in 0.1% PBST for 30
minutes at room temperature. The blots were then probed with the following antibodies:
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rabbit anti-human integrin α2 (1:1000), mouse anti-human integrin α1 (FB12, 1:1000), mouse anti-human integrin β1 (P4C10, 1:1000), mouse anti-human phosphotyrosine (P-Tyr-100, 1:2000), mouse anti-human phospho-FAK (14/FAK(Y397), 1:4000), rabbit anti-human FAK (polyclonal, 1:2000), mouse anti-human β-tubulin (D66, 1:6500) overnight at 4°C. After being washed in 0.1% PBST, the blots were correspondingly incubated with the second antibodies for 45 minutes at room temperature: anti-rabbit HRP (1:2000), anti-mouse HRP (1:2000), anti-mouse HRP (1:2000), anti-mouse HRP (1:4000), anti-mouse HRP (1:8000), anti-rabbit HRP (1:4000), anti-mouse HRP (1:10000). After being washed in 0.1% PBST, the blots were incubated with enhanced chemiluminescence solutions in dark for 5 minutes. The proteins were then visualized by an exposure to X-ray films.

To detect total FAK in the samples as a loading control (Fig. 23), the blots of pFAK were stripped by incubation in stripping buffer at 50°C for 50 minutes. After being washed in 0.1% PBST, the blots were reprobed with FAK polyclonal antibody (1:1000) and then with anti-rabbit HRP (1:2000), following the same procedures used for detection of pFAK.

2.2.11 Fluorescence microscopy

Cells were seeded on glass coverslips (with or without pre-coating of adhesive molecules) in the presence of SFM or medium containing 10% FBS or 50% PSC-SN. After 3 hours, cells were washed with PBS, fixed with 4% PFA for 20 minutes at room temperature, and washed again with PBS. The coverslips were then rinsed in ddH2O, air-dried, mounted on glass slides (2-3 slips per slide), and placed in a humid chamber. Cells were permeabilized with 0.2% Triton X-100/PBS for 10 minutes, and washed with 0.05% PBST. Nonspecific binding was blocked with TNB buffer for 30 minutes. For double staining of paxillin/pFAK plus F-actin, following steps were performed sequentially: 1) Incubation with primary antibodies for 1 hour, including mouse anti-human paxillin (5H11, 1:100), or mouse anti-human pFAK (14/FAK(Y397), 1:100); 2) incubation with the secondary antibody (rabbit-anti-mouse IgG-AlexaFluor 594, 1:100) for 45 minutes; 3) incubation with phalloidlin-AlexaFluor 488 (1:200) for 30 minutes. The nuclei were finally counter stained with Hoechst 33258 (2 µg/ml). TNB buffer was used for the dilution of antibodies. Each incubation step was followed by washing in 0.05% PBST 3 times for totally 15 minutes. Digital fluorescence images were obtained by epifluorescence microscopy.
2.2.12 Statistical analysis

The data were presented as means ± standard error of the mean (S.E.M.). To compare the difference between the control and test groups, statistical significance was assessed by one-way ANOVA with a Fisher’s LSD post hoc test. Two-tailed Student’s t test was used for the comparison between two independent groups. Significant difference was defined as $P < 0.05$. 
3. RESULTS

3.1 Effect of PSC-SN on the trans-migration of PCCs

To investigate the effect of PSCs on the trans-migration of Panc1 and UlaPaCa cells, different dilutions of PSC-SN were filled in the lower compartment of modified Boyden chamber. SFM and 10% FBS were used as the negative and positive control respectively. As shown in Fig. 2, PSC-SN induced the trans-migration of Panc1 and UlaPaCa cells in a dose-dependent manner. This stimulatory effect of PSC-SN was already significant at a concentration of 25%. A maximum effect was observed by 75% PSC-SN, which was comparable to that induced by 10% FBS. 50% PSC-SN was mainly used in the following experiments.

![Fig. 2: Effect of PSC-SN on the trans-migration of PCCs in modified Boyden chamber assay.](image)

3.2 Effect of PSC-SN on the adhesion and motility of PCCs

Cell trans-migration is a comprehensive process, mainly involving four biological events, i.e. cell adhesion on the microporous membrane, random motility, deformation across the membrane, and directionality of migration (chemotaxis/haptotaxis). To examine the effect of PSC-SN on the former two key events, cell adhesion assay and single cell tracking assay were employed.
3.2.1 PCC adhesion

As shown in Fig. 3, 50% PSC-SN facilitated the adhesion of Panc1 and UlaPaCa cells on plastic culture plates. Compared with SFM, the number of adherent cells in the presence of 50% PSC-SN was 2.2-fold higher for Panc1 (P < 0.05), and 1.8-fold higher for UlaPaCa (P < 0.05). The stimulatory effect of PSC-SN was even stronger than that of 10% FBS, with a statistical significance observed in Panc1 cells.

Fig. 3: Effect of PSC-SN on the adhesion of PCCs. Panc1 and UlaPaCa cells were seeded in 24-well plates containing SFM or 10% FBS or 50% PSC-SN, and allowed to adhere for 1 hour. The adherent cells were identified as those remained after being washed with PBS and stained with Hoechst 33258, and were counted from 9 random fields. Results are expressed as the mean ± S.E.M. of three independent experiments. * P < 0.05 compared with corresponding SFM controls. & P < 0.05 compared with 10% FBS.

3.2.2 PCC motility

To examine the effect of PSC-SN on the motility of Panc1 and UlaPaCa cells, single cell tracking assays were performed with two protocols (Fig. 4-middle).
RESULTS

Fig. 4: Effect of PSC-SN on the motility of PCCs. Schematic depiction of two approaches in single cell tracking assays is shown in the middle. In protocol a, Panc1 and UlaPaCa cells were seeded in medium containing 10% FBS and cultured for 24 hours, followed by 12-hour starvation in SFM (to slow down baseline cell motility), and then stimulated by 50% PSC-SN or 10% FBS or SFM as a control for 3 hours. Cell migration during the next 24 hours was recorded by the time-lapse microscopy. In protocol b, PCCs were directly seeded in SFM or medium containing 10% FBS or 50% PSC-SN and allowed to adhere for 1 hour. Cell movement was then recorded over 24 hours. More than 30 cells were randomly taken for manual tracking by ImageJ 1.44m. Cell velocity (µm/h) was calculated from the trace of each cell and analyzed in Excel. Results are expressed as the mean ± S.E.M. of three independent experiments. * P < 0.05 compared to corresponding SFM controls.

On the left panel of Fig. 4, PSC-SN slightly stimulated the random motility of both cell lines (relative to SFM: 1.9-fold increase in Panc1, 1.6-fold in UlaPaCa). However, compared with the stimulation from 10% FBS (P < 0.05 relative to SFM: 4.2-fold increase in Panc1, 4.0-fold in UlaPaCa), the stimulatory effect of PSC-SN was much weaker (P < 0.05). On the right panel of Fig. 4, PSC-SN significantly accelerated the velocity of both cell lines (P < 0.05 compared with SFM: 7.2-fold increase in Panc1, 6.1-fold increase in UlaPaCa). Moreover, the stimulation of PSC-SN was comparable to that of 10% FBS.

The essential difference between the two protocols is that cell adhesion is mediated by different media. In protocol a, PCCs were adhered by 10% FBS, and PSC-SN was used as a pure motility-stimulator upon readily adherent cells. In protocol b, the addition of
PSC-SN to PCCs was concomitant with cell seeding, so that PSC-SN affected both the adhesion and motility. The tremendously increased PCC velocity in protocol b suggests that PSC-SN-mediated adhesion is crucial for its subsequent effect on motility. Acceleration of cell motility by PSC-SN appears to be critically dependent on specific adhesive molecules (e.g. matrix proteins) rather than soluble factors (e.g. cytokines).

3.3 Role of adhesive molecules versus soluble factors in PSC-SN-induced PCC trans-migration

To figure out whether the stimulation of PSC-SN on PCC trans-migration is via adhesive molecules or via soluble factors, two sets of modified Boyden chamber assays were designed.

In the first experiment (Fig. 5-left panel), one group of inserts (group i) was pre-incubated for 1 hour with SFM or 10% FBS or 50% PSC-SN, allowing the adhesive molecules in media to be coated onto the underside of inserts. The other group of inserts (group ii) was left without pre-treatment of the media before cell seeding.

As shown in Fig. 5 (right panel), pronounced amount of Panc1 and UlaPaCa cells in both groups trans-migrated towards 10% FBS, which is broadly used as a positive chemoattractant. Interestingly PSC-SN comparably induced the trans-migration of PCCs in group i, but showed little stimulation on cells in group ii. This observation indicates that 1) the pre-coating of inserts with adhesive molecules in PSC-SN is required for PSC-SN-promoted trans-migration of PCCs; 2) the soluble factors in PSC-SN are less efficient compared with those in 10% FBS to induce PCC chemotaxis. These two points were further verified in the next set of experiment.
**Fig. 5: Effect of PSC-SN on PCC trans-migration with or without pre-incubation of inserts.** Schematic illustration of the protocol for this experiment is shown on the left panel. The lower compartment of Boyden chamber was filled with SFM or medium containing 10% FBS or 50% PSC-SN. Group i insert was pre-incubated in the above media for 1 hour, while group ii was left outside till 1 hour later. Panc1 and UlaPaCa cells in SFM were seeded into the inserts and allowed to trans-migrate for 18 hours. Representative images for each condition are shown. Scale bar: 200 µm.

As illustrated in Fig. 6 (upper panel), by exchanging the lower chambers after coating of the inserts, adhesive molecules and soluble factors in PSC-SN were supposed to be separated into two chamber systems. Consequently, PCC trans-migration through the PSC-SN-coated inserts was significantly induced even in the absence of any cytokines in lower compartment (Fig. 6-lower panel). Without coating of the inserts, however, few cells trans-migrated towards PSC-SN that is ‘depleted’ of adhesive molecules and used as a chemoattractant. This observation suggests a strong haptotactic/haptokinetic effect but a faint chemotactic effect of PSC-SN on PCCs.
Fig. 6: Effect of pre-coating of inserts with PSC-SN on PCC trans-migration. Schematic illustration of the protocol for this experiment was shown on the upper panel. Inserts were placed into lower chambers containing SFM or 50% PSC-SN and incubated for 1 hour. Thereafter the lower chambers were exchanged, so that PSC-SN-coated inserts placed into SFM whereas SFM-embedded inserts into PSC-SN. Panc1 and UlaPaCa cells were seeded and allowed to trans-migrate for 18 hours. Representative images for each condition are shown. Scale bar: 200 µm.

3.4 Effect of ECM proteins on the adhesion of PCCs

The stroma in PDAC consists of various ECM proteins including collagen (type I, III, IV), fibronectin, laminin, and vitronectin, with collagen I and fibronectin to be the most abundant (Gress et al. 1995; Linder et al. 2001; Ryschich et al. 2009; Shimoyama et al. 1995). On the other side, PSCs are proven to produce collagen I, III, fibronectin (Bachem et al. 2005) and laminin (Jesnowski et al. 2005). To verify which adhesive molecule was mainly responsible for PSC-SN-mediated cell adhesion, the effect of collagen I, fibronectin or laminin on the adhesion of Panc1 and UlaPaCa cells was examined.

3.4.1 Collagen I and fibronectin
After coating culture wells with collagen I, the adhesion of Panc1 and UlaPaCa cells in the presence of SFM, or 10% FBS, or 50% PSC-SN were all improved and even comparable to each other (Fig. 7). Improved adhesion was most significant for cells in SFM on collagen I (P < 0.05 compared with SFM w/o coating, 7.3-fold increase in Panc1, 12-fold in UlaPaCa). Fibronectin showed no further stimulatory effect on PCC adhesion.

**Fig. 7: Effect of collagen I and fibronectin on the adhesion of PCCs.** 24-well plates were left uncoated or were coated with collagen I (Col) and/or fibronectin (Fn) as described in the method section. Panc1 and UlaPaCa cells were then seeded in SFM or medium containing 10% FBS or 50% PSC-SN. After 1 hour, adherent cells were stained with Hoechst 33258, photographed and counted in 9 random fields. Results are expressed as the mean ± S.E.M. fold of 10% FBS w/o coating of three independent experiments. * P < 0.05.

### 3.4.2 Dose-effect assessment of collagen I

Collagen I showed a biphasic effect on the adhesion of Panc1 and UlaPaCa. In the range of 760 ng/ml-10 µg/ml, collagen I showed the strongest stimulation on cell adhesion, which was comparable to the effect of 50% PSC-SN (Fig. 8). In most following studies, collagen I at 1 µg/ml was used for the coating of culture surfaces.
**Fig. 8: Dose-effect assessment of collagen I in PCC adhesion.** 24-well plates were left uncoated or were coated with collagen I (Col) at varying concentrations. Panc1 and UlaPaCa cells were then seeded in SFM (with or w/o coated Col), or in medium containing 10% FBS or 50% PSC-SN. After 1 hour, adherent cells were stained with Hoechst 33258, photographed and counted in 9 random fields. Results are expressed as the mean ± S.E.M. fold of 10% FBS w/o coating of three independent experiments.

### 3.4.3 Laminin

Laminin, at a relatively low dose (7.6-76 ng/ml), showed a slight stimulation on the adhesion of Panc1 and UlaPaCa, but with no statistical significance compared with SFM (Fig. 9). At the concentration of 10 µg/ml, laminin obviously inhibited the adhesion of both cell lines.
Fig. 9: Dose-effect assessment of laminin in PCC adhesion. 24-well plates were left uncoated or were coated with laminin at varying concentrations. Panc1 and UlaPaCa cells were then seeded in SFM (with or w/o coated laminin), or in medium containing 10% FBS or 50% PSC-SN. After 1 hour, adherent cells were stained with Hoechst 33258, photographed and counted in 9 random fields. Results are expressed as the mean ± S.E.M. fold of 10% FBS w/o coating of three independent experiments.

3.4.4 Effect of a RGD peptide on PSC-SN- or collagen I-mediated PCC adhesion
To further verify whether fibronectin is involved in PSC-SN mediated PCCs adhesion, Panc1 and UlaPaCa cells were pre-treated with a RGD peptide, which contains the integrin-recognition sequence present in ECM proteins such as fibronectin and vitronectin but not collagens (Barczyk et al. 2010; Humphries et al. 2006). As shown in Fig. 10, neither PSC-SN- nor collagen I-mediated cell adhesion was influenced by the RGD peptide.
Fig. 10: Effect of a RGD peptide on PSC-SN- or collagen I-facilitated PCC adhesion. Cells were pre-incubated with RGD peptide (100 µg/ml or 200 µg/ml) for 1 hour. The cells were then seeded in SFM or medium containing 10% FBS or 50% PSC-SN (SN), with or without pre-coated collagen I (Col, 10 µg/ml). After 1 hour, adherent cells were determined as previously described. Results are expressed as the mean ± S.E.M. number of adherent cells from three independent experiments.

As a positive control for RGD peptide, we applied the melanoma cell line A375 which expresses integrin αvβ3 and can adhere to RGD-containing protein such as fibronectin (Trabocchi et al. 2010). As shown below, RGD peptide dose-dependently reduced fibronectin- and PSC-SN-induced A375 adhesion (Fig. 11).

Fig. 11: Effect of a RGD peptide on fibronectin- or PSC-SN-facilitated A375 cell adhesion. Cells were pre-incubated with RGD peptide (100 µg/ml or 200 µg/ml) for 1 hour. The cells were then seeded in SFM or medium containing 50% PSC-SN (SN), with
RESULTS

3.5 Effect of adhesive molecules on the motility of PCCs

To verify which adhesive molecule was mainly responsible for PSC-SN-stimulated cell migration, the effect of collagen I or fibronectin on the motility of Panc1 and UlaPaCa cells was assessed by single cell tracking assay.

3.5.1 Collagen I

PCCs were seeded in the presence of SFM, or 10% FBS, or 50% PSC-SN with or without pre-coated collagen I. After 1 hour adhesion, the random cell migration was recorded over 24 hours. Compared with non-coated conditions, pre-coated collagen I significantly accelerated the motility of PCCs in SFM (Panc1: 10.5 vs 23.4 µm/h; UlaPaCa: 10.5 vs 23.5 µm/h; P < 0.05), as well as in 10% FBS (Panc1: 27.3 vs 54.9 µm/h; UlaPaCa: 26 vs 46 µm/h; P < 0.05). In medium containing 50% PSC-SN, there was no further stimulation after coating of collagen I, but the cell velocity was still significantly higher than that in SFM with coating (Fig. 12).

These data indicate that 1) collagen I alone is sufficient to stimulate cancer cell motility by providing a substratum; 2) collagen I presented in PSC-SN is sufficient to stimulate PCC motility; 3) besides collagen I, other factors in PSC-SN act synergistically to promote the motility of PCCs.

Fig. 12: Effect of collagen I on the motility of PCCs. Panc1 and UlaPaCa cells were...
RESULTS

seeded in SFM or medium containing 10% FBS or 50% PSC-SN (SN), with or without pre-coated collagen I (Col, 1 µg/ml). After 1 hour adhesion, migration of adherent cells were recorded over 24 hours by the time-lapse microscopy, and cell velocity was analyzed as described before. Results are expressed as the mean ± S.E.M. of three independent experiments. * P < 0.05 compared with w/o coating SFM control; ** P < 0.05 compared with collagen I coating SFM control; & P < 0.05.

3.5.2 Poly-L-lysine
To examine whether the acceleration of PCCs motility is ligand-dependent, poly-L-lysine was used as a non-specific adhesive molecule which improves cell adhesion through the interaction of electric charges rather than ligand-receptor. At the concentration of 22 µg/ml, poly-L-lysine significantly improved the adhesion of Panc1 and UlaPaCa cells in SFM. This stimulatory effect was comparable to that of 50% PSC-SN (Fig. 13A). Based on the improved adhesion, however, poly-L-lysine showed no further stimulation on the subsequent motility of PCCs (Fig. 13B).

Fig. 13: Effect of poly-L-lysine on the adhesion and motility of PCCs. (A) 24-well plates were left uncoated or were coated with poly-L-lysine (PLL) at 22µg/ml. Panc1 and UlaPaCa cells were then seeded in SFM (with or w/o coated PLL), or in medium containing 10% FBS or 50% PSC-SN. After 1 hour, adherent cells were stained with Hoechst 33258, photographed and counted in 9 random fields. Results are expressed as the mean ± S.E.M. fold of 10% FBS w/o coating of three independent experiments. * P < 0.05. (B) Panc1 and UlaPaCa cells were seeded in SFM or medium containing 10% FBS or 50% PSC-SN (SN), with or without pre-coated PLL. After 1 hour adhesion, migration of adherent cells were recorded over 24 hours by the time-lapse microscopy, and cell velocity...
was analyzed as described before. Results are expressed as the mean ± S.E.M. of three independent experiments. * P < 0.05 compared with w/o coating SFM control; ** P < 0.05 compared with collagen I coating SFM control.

3.5.3 Fibronectin

To compare the efficiency of different substrata on the stimulation of PCCs motility, culture wells were coated with fibronectin, collagen, or poly-L-lysine as a control. Panc1 and UlaPaCa cells were then seeded with 10% FBS which was used to facilitate cell attachment. After 1 hour, the culture medium was changed to SFM after two times of washing with PBS so that non-adherent cells and various factors from FBS were removed. Random cell migration over 30 hours was recorded. As shown in Fig. 14, the average velocity of PCCs on fibronectin was similar to that on poly-L-lysine, and was significantly slower than the cell velocity on collagen I.

![Graph showing cell velocity comparison](image)

**Fig. 14: Effect of matrix proteins on the motility of PCCs based on 10% FBS facilitated adhesion.** 24-well plates were pre-coated with poly-L-lysine (PLL, 22 μg/ml), collagen I (Col), or fibronectin (Fn). Panc1 and UlaPaCa cells were seeded with 10% FBS and allowed to adhere for 1 hour. Afterward the culture plates were washed twice with PBS and SFM were filled in. Cell migration was recorded over 30 hours by the time-lapse microscopy, and cell velocity was analyzed. Results are expressed as the mean ± S.E.M. of three independent experiments. * P < 0.05 compared with corresponding poly-L-lysine coating SFM controls.

3.6 Effect of adhesive molecules on the trans-migration of PCCs

To compare the effect of different adhesive molecules on PCC trans-migration, the underside of inserts was coated with collagen I, fibronectin or poly-L-lysine. As shown in
Fig. 15A, collagen I significantly induced the trans-migration of Panc1 and UlaPaCa cells, even when there was merely SFM in the lower compartment. This stimulatory effect was equivalent to that of 50% PSC-SN. However, neither poly-L-lysine nor fibronectin induced cell trans-migration. Moreover, neither collagen I- nor PSC-SN-induced cell trans-migration was affected by RGD peptide (Fig. 15B), which further excluded the involvement of fibronectin.

![Image](image-url)

**Fig. 15: (A) Effect of matrix proteins on PCC trans-migration.** The lower compartment of Boyden chamber was filled with SFM or medium containing PSC-SN. Panc1 and UlaPaCa cells were seeded into the inserts which had been coated or not coated (w/o) with poly-L-lysine (PLL, 22 µg/ml) or fibronectin (Fn, 10 µg/ml) or collagen I (Col, 1 µg/ml) on the underside as described in the method. After 18 hours, the trans-migrated cells were stained with Hoechst 33258 and photographed (100×). **(B) Effect of RGD on collagen I/PSC-SN-induced Panc1 trans-migration.** Panc1 cells, which had been pre-incubated with 200 µg/ml RGD peptide for 1 hour, were applied to the modified Boyden Chamber assay as described above. Representative images for each condition are shown. Scale bar:
3.7 Effect of PSC-SN/collagen I on the directionality of PCC trans-migration

To evaluate the directionality of PCCs during trans-migration, the inserts were coated with collagen I or PSC-SN either on the underside or on both sides, while the lower chambers were filled with SFM.

Compared with w/o coating (image a, f, Fig. 16A), collagen I or PSC-SN coated on both sides (image b, d, g, i) significantly induced the trans-migration of Panc1 and UlaPaCa, indicating that haptokinesis of both cell lines were stimulated. Based on this effect, a further stimulation on PCCs trans-migration was observed when collagen I or PSC-SN was coated on the underside (image c, e, h, j). This further stimulation based on the haptokinesis represents the haptotaxis of PCCs.

Quantification of trans-migrated cells is shown in Fig. 16B. For Panc1 cells stimulated by collagen I, the cell number in b is 348/field, which stands for haptokinesis taking up 77% of the cell number (455/field) in c; the other 23% in c represents haptotaxis. Accordingly, haptotaxis accounts for 46% of collagen I-induced Panc1 trans-migration. In UlaPaCa cells, however, haptotaxis constitutes 60% and 80% of collagen I- and PSC-SN-induced trans-migration, respectively. In conclusion, PSC-SN or collagen I mainly promoted the haptokinesis of Panc1 but haptotaxis of UlaPaCa.
Fig. 16: Effect of PSC-SN or collagen I on the directionality of PCC trans-migration. (A) Inserts without coating (a, f) were used as a negative control. Other inserts were coated with collagen I (Col, 1 µg/ml) or PSC-SN either on both sides (b, g; d, i) or on the underside (c, h; e, j) as described in the method. Lower chambers were filled with SFM. Panc1 and UlaPaCa cells were allowed to trans-migrate for 18 hours. Representative images for each condition are shown. (B) Quantification of PCC trans-migration. Images were taken from 7 random fields and the cell number was counted with ImageJ 1.44m. Scale bar: 200 µm.

3.8 Expression of integrin subunits on PCCs
The major and best-characterized receptors mediating ECM-cell interactions are integrins, which are heterodimeric trans-membrane proteins composed of non-covalently associated \( \alpha \) and \( \beta \) subunits (Hynes 2002). Integrin ligand specificity is determined by the \( \alpha \) subunit, whereas the \( \beta \) subunit is connected to cytoskeleton and initiates intracellular signaling...
RESULTS

Among the 24 different α-β combinations, collagens are recognized by integrins α1β1, α2β1, α10β1 and α11β1, with the former two most widely studied (Hynes 2002).

Western blot was used to analyze integrin expression in Panc1, UlaPaCa as well as two other cell lines MiaPaCa-2 und AsPC-1. Panc1 and UlaPaCa cells abundantly expressed the α2 and β1 subunits; MiaPaCa-2 expressed less α2; AsPC-1 presented a very faint band of β1 subunit (Fig. 17). In accordance with the data of Grzesiak et al (Grzesiak and Bouvet 2006), none of the tested cells expressed the α1 subunit (Fig. 17). Thus integrin α2β1 represents the major collagen I receptor on Panc1 and UlaPaCa cells.

![Western blot analysis for the expression of α1, α2 and β1 integrin subunits in PCCs. 30 µg of total cell lysates from Panc1, UlaPaCa (Ula), MiaPaCa-2 (Mia), and AsPC-1 cultured with 10% FBS for 24 hours were subjected to 8% SDS-PAGE. Antibodies against human α1, α2 and β1 integrin subunits were used to detect the corresponding expression. The band of PSCs (arrow) is shown as a positive control for α1 integrin. β-tubulin was used as a loading control.](image)

3.9 Effect of anti-integrin antibodies on PSC-SN/collagen I-induced adhesion and trans-migration of PCCs

3.9.1 Effect of anti-integrin α2β1 antibody on the induced PCC adhesion

The contribution of collagen I to PSC-SN-induced cell adhesion was examined with a blocking antibody against integrin α2β1. Adhesion of both cells on collagen I was completely blocked by anti-integrin α2β1 antibody (Fig. 18). Although not completely, PCCs adhesion in the presence of PSC-SN was largely attenuated (59% decrease in Panc1 and 66% in UlaPaCa), indicating that collagen I is the major mediator for PSC-SN-promoted cell adhesion via integrin α2β1.
Fig. 18: Effect of integrin $\alpha 2\beta 1$ blocking antibody on PSC-SN- or collagen I-induced PCC adhesion. Panc1 and UlaPaCa cells were pre-incubated with 5 $\mu$g/ml integrin $\alpha 2\beta 1$ blocking antibody for 1 hour, and then were allowed to adhere for 1 hour in SFM (with or w/o coated collagen I (Col)) or medium containing 50% PSC-SN. Results are expressed as the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ compared to corresponding controls w/o antibodies.

3.9.2 Effect of anti-integrin $\alpha 2$ or/and $\beta 1$ antibodies on the induced PCC trans-migration

To further examine whether collagen I is virtually responsible for PSC-SN induced PCCs trans-migration, blocking antibodies against $\alpha 2$ and/or $\beta 1$ subunits were used in modified Boyden chamber assay. Anti-integrin $\alpha 2$ antibody abolished the haptotactic trans-migration of UlaPaCa induced by collagen I, but showed a limited blocking effect against stimulation from PSC-SN (Fig. 19). In contrast, anti-integrin $\beta 1$ antibody significantly inhibited PSC-SN-induced haptotaxis of UlaPaCa. This inhibition became even more obvious after combined utilization of anti-integrin $\alpha 2$ and $\beta 1$ antibodies. These data show that collagen I is the major component in PSC-SN that mediates the stimulatory effect on PCCs via integrin $\alpha 2\beta 1$. 
Fig. 19: Effect of blocking antibodies against integrin α2 or/and β1 on PSC-SN- or collagen I-induced UlaPaCa trans-migration. UlaPaCa cells were pre-incubated with 20 µg/ml integrin α2 or/and β1 blocking antibodies for 1 hour. The cells were then seeded into inserts (with or w/o coated collagen I (Col) on the underside) which had been placed in lower chambers containing SFM or 50% PSC-SN plus corresponding blocking antibodies. After 18 hours, the trans-migrated cells were stained with Hoechst 33258 and photographed. Representative images for each condition are shown. Scale bar: 200 µm.

3.10 Effect of PSC-SN/collagen I on the motile morphology of PCCs

3.10.1 PCC morphology observed by bright-field microscopy

In adhesion assay, both PSC-SN and collagen I obviously promoted the spreading of PCCs, whereas cells seeded in SFM or on poly-L-lysine were merely small and round (Fig. 20). Moreover, compared with PCCs spreading in FBS which were circumferential or polygonal, cells in the presence of PSC-SN or on collagen I were elongated and polarized with the extension of lamellipodia.

Fig. 20: Morphology of PCCs adhered under distinct conditions. Panc1 and UlaPaCa cells were seeded in medium containing 10% FBS or 50% PSC-SN (w/o coating), or in SFM (with or w/o coated poly-L-lysine (PLL) or collagen I (Col)). After 1 hour, phase contrast images were taken. Representative images for each condition are shown. Scale bar:
100 µm.

In single cell tracking assay, PCC migration on collagen I or in medium containing PSC-SN was a multistep cycle, which began with the protrusion of membrane at cell front, followed by translocation of cell body, and finally the release and traction of cell rear. For PCCs in SFM or on poly-L-lysine, most were immobile with no extension of cell membrane, while a few cells extended multiple protrusions at a time and showed an oscillatory movement within short distance.

PSC-SN and collagen I induced resembling motile morphology of PCCs, which was consistent with their comparable stimulations on cell adhesion and migration. This indicates that common intracellular event(s) might be initiated by PSC-SN and collagen I.

3.10.2 F-actin and focal adhesions examined by fluorescence microscopy

To figure out the intracellular mechanism related to cell spreading and polarization, fluorescence stainings of F-actin, pFAK and paxillin were performed (Fig. 21). For cells stimulated by PSC-SN or collagen I, a strong formation of F-actin in the cell body and actin polymerization in the lamellipodia were clearly observed. Moreover, pFAK and paxillin, two of the major scaffold proteins in focal adhesions (FAs) (Schlaepfer et al. 2004), were significantly distributed on the periphery of PCCs. For cells in SFM or on poly-L-lysine, however, pFAK and paxillin were aggregated within the cytoplasm, and FAs were hardly observed. These data imply that PSC-SN and collagen I initiated the coordinated and dynamic regulation of FAs and cytoskeleton networks, which is required for efficient cell migration.
Panc1 cells
(Fig. 21, see the legend on page 46)
UlaPaCa cells.
(Fig. 21, see the legend on page 46)
Fig. 21: Fluorescence stainings of pFAK, paxillin and F-actin in PCCs. Glass coverslips were placed in 6-well plates and coated with 22 μg/ml poly-L-lysine (PLL) or 10 μg/ml collagen I (Col). Panc1 and UlaPaCa cells were seeded in SFM (with or w/o coating) or medium containing 50% PSC-SN (w/o coating). After 3 hours, non-adherent cells were washed away by PBS, while adherent cells were fixed by 4% PFA for 20 minutes at room temperature. Antibodies against pFAK (Tyr397, red) and paxillin (red) were used to detect the distribution of focal adhesion complex proteins. F-actin stained with rhodamine-phalloidin (green) was used for the observation of cell cytoskeleton. Nuclei were stained with Hoechst 33258 (blue). In each image, the boxed region is shown magnified in the insert. Arrows indicate representative focal adhesions, and asterisks indicate representative lamellipodia. Scale bars: 40 μm.

3.11 Effect of PSC-SN/collagen I on the protein phosphorylation in PCCs

To further clarify the molecular mechanisms involved in PSC-SN/collagen I-induced haptokinesis of PCCs, a time-course analysis of phosphotyrosine was performed by Western blot. Cell lysates were collected at sequential time points after cell seeding in the presence of SFM, poly-L-lysine, collagen I, or PSC-SN. As shown in Fig. 22, several proteins with different molecular weights were phosphorylated at the tyrosine site(s). Interestingly, PSC-SN or collagen I induced a constant and strong phosphorylation of the proteins between 100 kDa and 150 kDa. For cells seeded in SFM or on poly-L-lysine, a transient phosphorylation was observed within 10 minutes, but was obviously attenuated thereafter, indicating a non-specific stimulation from stress or mechanical performance (e.g. centrifugation, pipette).
RESULTS

Fig. 22: Effect of PSC-SN or collagen I on the phosphorylation of tyrosine sites in a time course study of Western blot. Panc1 and UlaPaCa cells were seeded at 80% confluence in 6-well plates containing SFM (with or w/o coated poly-L-lysine or collagen I) or 50% PSC-SN (w/o coating). The culture was stopped at indicated time points after cell seeding. Both non-adherent and adherent cells were collected and lysed. 16-18 µg of total cell lysates were subjected to 6% SDS-PAGE, and phosphotyrosine was used to detect the phosphorylation of tyrosine sites in proteins. The same blots were re-probed with antibody against total FAK as loading controls (SFM, poy-L-lysine, collagen I, 50% PSC-SN).

Because FAK is one of the key regulatory proteins for cell migration (Schlaepfer et al. 2004), we speculated that FAK might be involved in PSC-SN/collagen I-induced PCC haptokinesis. A time-course Western blot analysis was used to examine the phosphorylation of FAK (Tyr397). Just 10 minutes after seeding (Fig. 23A), PSC-SN or collagen I strongly stimulated a phosphorylation of FAK in Panc1 cells, which remained at a significantly higher level than cells in SFM during the following time period. For cells seeded on poly-L-lysine, FAK phosphorylation was also increased within 10 minutes, which was due to a mechanical stress. UlaPaCa cells showed similar results to Panc1 cells, as shown in Fig. 23B. Interestingly, the levels of pFAK were consistent with the pattern of cell velocity under different conditions in single cell tracking assay (Fig. 23C). Corresponding to the continuously enhanced phosphorylation of FAK by PSC-SN or collagen I, PCC motility in these two conditions was constantly higher than that in SFM or poly-L-lysine.
Fig. 23: Effect of PSC-SN or collagen I on the phosphorylation of FAK in a time course study of Western blot. (A, B) Panc1 (A) and UlaPaCa (B) cells were seeded at 80% confluence in 6-well plates containing SFM (with or w/o coated poly-L-lysine or collagen I) or 50% PSC-SN (w/o coating). The culture was stopped at indicated time points after cell seeding. Both non-adherent and adherent cells were collected and lysed. 12 µg of total cell lysates were subjected to 6% SDS-PAGE, and pFAK (Tyr397) was used to detect the activation of this kinase. The same blots were reprobed with antibody against total FAK as loading controls (① SFM, ② poy-L-lysine, ③ collagen I, ④ 50% PSC-SN).
Quantification of pFAK was performed by scanning densitometry from three independent experiments, and presented as mean ± S.E.M. fold increase above w/o coating SFM. * P<0.05. (C) Cell velocity profiles under the above conditions were obtained from 24-hour single cell tracking assay.

3.12 Effect of FAK inhibition on PSC-SN/collagen I-induced adhesion and migration of PCCs

To further examine the functional relevance of increased FAK phosphorylation to PCC haptokinesis and haptotaxis, the FAK inhibitor PF-537228 and siRNA-mediated FAK silencing were employed to disrupt the function of FAK.

3.12.1 Inhibition of FAK phosphorylation by PF-573228

3.12.1.1 Effect of PF-573228 on PCC trans-migration

After treatment with PF-537228 (Fig. 24), PSC-SN- or collagen I- induced haptokinesis of Panc1 and haptotaxis of UlaPaCa was significantly inhibited, whereas FBS-stimulated chemotaxis of both cell lines was not affected.

Fig. 24: Effect of a FAK inhibitor (PF-537228) on PSC-SN- or collagen I-induced PCC trans-migration. Panc1 and UlaPaCa cells were pre-incubated with 1 µM PF-537228 (PF) for 1 hour. DMSO (0.1%), the dissolvent for PF-537228, was used as a negative control. Afterward the cells were seeded into inserts (with or w/o coated collagen I (Col) on the underside) which had been placed in lower chambers containing SFM, 10% FBS, or 50% PSC-SN plus DMSO or PF-537228. After 18 hours, the trans-migrated cells were stained with Hoechst 33258 and photographed. Representative images for each condition are shown. Scale bar: 200 µm.
3.12.1.2 Effect of PF-573228 on PCC motility

Random motility is one of the key events involved in cell trans-migration. Treatment of PCCs with PF-537228 before seeding them on collagen I or in medium containing PSC-SN (Fig. 25) significantly attenuated the random cell motility.

![Graph showing effect of PF-573228 on PCC motility](image)

**Fig. 25: Effect of a FAK inhibitor (PF-537228) on PSC-SN- or collagen I-stimulated PCC motility.** Panc1 and UlaPaCa cells pre-incubated with 0.1% DMSO or 1 μM PF-537228 were seeded in SFM (with or w/o coated collagen I (Col)) or medium containing 50% PSC-SN. After 1 hour adhesion, cell migration was recorded over 24 hours and the cell velocity was analyzed as described before. Results are expressed as the mean ± S.E.M. from three independent experiments. * P < 0.05 compared with corresponding 0.1% DMSO controls.

3.12.1.3 Effect of PF-573228 on PCC adhesion

Cell adhesion is the basis of migration. Unsurprisingly, PF-537228 slightly decreased the number of adherent cells on collagen I or in medium containing PSC-SN, with about 15% and 35% decrease, respectively (Fig. 26).
Fig. 26: Effect of a FAK inhibitor (PF-537228) on PSC-SN-or collagen I-induced PCC adhesion. Panc1 and UlaPaCa cells pre-incubated with 0.1% DMSO or 1 µM PF-537228 were seeded in SFM (with or w/o coated collagen I (Col)) or medium containing 50% PSC-SN. After 1 hour, adherent cells were stained, photographed and counted at 9 random fields. Results are expressed as the mean ± S.E.M. fold of 10% FBS plus 0.1% DMSO of three independent experiments. * P < 0.05 compared with corresponding 0.1% DMSO controls.

3.12.2 Inhibition of FAK expression by siRNAs
Because PF-573228 is a pharmological inhibitor which has the potential for non-specific or off-target effects, siRNA-mediated silencing of FAK was employed to further examine the involvement of this kinase in PSC-SN/collagen I-induced PCC hapto-migration.

3.12.2.1 siRNA-mediated knockdown of FAK
To find out the time point by which FAK expression and activity is decreased by siRNAs, a time course Western blot was performed in transfected Panc1 cells. 48 hours post-transfection, the level of FAK started to decrease; after another 18 hours, the silencing of FAK expression became more obvious (Fig. 27). The phosphorylation of FAK was also reduced 66 hours post-transfection, however the decline of pFAK was less significant than that of FAK.
**RESULTS**

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**Fig. 27:** A time course Western blot analysis of FAK phosphorylation and expression during siRNA-mediated gene silencing. Panc1 cells were seeded at 70% confluence with 10% FBS for 1 hour, and then were transfected with 40 nM siRNAs using HiPerFect Transfection Reagent. At indicated time points after transfection, cells were collected and lysed. 12 µg of total cell lysates were subjected to 8% SDS-PAGE. Anti-pFAK (Tyr397) and anti-FAK were used to detect the phosphorylation and the expression of this kinase, respectively. Anti-β-tubulin was used as a loading control (① Neg siRNA, ② FAK siRNA1, ③ FAK siRNA2).

To improve the inhibitory efficacy of siRNAs, we modified the protocol by decreasing the cell confluency for transfection to 50% and changing media with 1% FBS 48 hours post-transfection. As shown in Fig. 28, siRNA-mediated gene silencing resulted in 40-65% reduction in the phosphorylation of FAK and over 80% reduction in the expression of FAK.
RESULTS

Fig. 28: Western blot analysis of FAK phosphorylation and expression 66 hours after siRNA transfection. Panc1 cells were seeded at 50% confluence with 10% FBS for 1 hour, and then were transfected with 40 nM siRNAs using HiPerFect Transfection Reagent. Media were changed 48 hours post-transfection and the cells were maintained in fresh medium containing 1% FBS for additional 18 hours. Afterward cell lysates were collected and subjected to 8% SDS-PAGE. Anti-pFAK (Tyr397) and anti-FAK were used to detect the phosphorylation and the expression of this kinase, respectively. Anti-β-tubulin was used as a loading control. Quantification of pFAK and FAK were performed by scanning densitometry from four independent experiments, and presented as mean ± S.E.M. percentage of Neg siRNA. * P < 0.05.

3.12.2.2 Effect of FAK siRNAs on PCC haptokinesis
After FAK knockdown by siRNAs, both collagen I- and PSC-SN-stimulated Panc1 motility (Fig. 29A) and trans-migration (Fig. 29B) were significantly attenuated.
Fig. 29: Effect of FAK siRNAs on PSC-SN- or collagen I-stimulated PCC haptokinesis. Panc1 cells were transfected with 40 nM negative siRNA, FAK siRNA1 or FAK siRNA2 for 66 hours. (A) Transfected cells were seeded in SFM (with or w/o coated collagen I (Col)) or medium containing 50% PSC-SN. After 1 hour adhesion, cell migration was recorded over 24 hours by the live-cell time-lapse microscopy, and the cell velocity was analyzed as described before. Results are expressed as the mean ± S.E.M. from three independent experiments. * P < 0.05 compared with corresponding Neg siRNA controls. (B) Transfected cells were seeded into inserts (with or w/o coated collagen I on the underside) which had been placed in lower chambers containing SFM or 50% PSC-SN. After 18 hours, the trans-migrated cells were stained with Hoechst 33258 and photographed (100×). Representative images for each condition are shown. Scale bar: 200 µm.
4. DISCUSSION

4.1 PSC-SN mainly induced the haptokinetic/haptotactic trans-migration of PCCs

Activated PSCs synthesize multiple cytokines and growth factors. Connective tissue growth factor (Eguchi et al. 2012), stromal cell-derived factor-1 (Li et al. 2012) and activin (Lonardo et al. 2012) are addressed in PSC-induced migration or invasion of pancreatic cancer cells. Besides, PSCs produce PDGF (Vonlaufen et al. 2008), VEGF (Xu et al. 2010), TGF-β1 (Shek et al. 2002), monocyte chemotactic protein-1 (Masamune et al. 2002) and COX-2 (Yoshida et al. 2005), which are reported to promote PCC proliferation, angiogenesis in PDAC, or autocrine activation of PSCs. Although not verified in PSC-induced PCC migration, these factors have been implicated in the chemotaxis of many cancer cells, including pancreatic cancer cells (Bartscht et al. 2012; Kawaguchi et al. 2012; Loberg et al. 2006; Vanderkerken et al. 2002; Wey et al. 2005).

In our experimental system, however, the conditioned medium from PSCs did not show a significant effect on the chemokinetic or chemotactic response of Panc1 and UlaPaCa cells. Instead, the haptokinesis or haptotaxis of PCCs were strongly induced by PSC-SN.

PSC-SN-induced PCC haptokinesis/haptotaxis is established on 2 major findings: 1) PSC-SN mediated cell adhesion was a prerequisite for the strong stimulation of the random motility of Panc1 and UlaPaCa. As a pure chemokinetic stimulator upon readily adherent cells, PSC-SN was much less powerful than 10% FBS in accelerating cell motility. 2) Pre-incubation of inserts in PSC-SN was necessary, as well as sufficient for its robust effect on the trans-migration of Panc1 and UlaPaCa. As a pure chemoattractant that ‘depleted’ of adhesive molecules, PSC-SN only weakly induced the cell trans-migration. Thus it is reasonable to deduce that PSC-SN promoted the trans-migration of PCCs by coating some adhesive molecule(s) onto the interface of the insert, which in turn facilitated cell adhesion and accelerated the followed motility. This kind of cell migration, closely associated with the substrate-bound molecules, coincides with the biology of haptokinesis/haptotaxis.

In mammary cancer, haptotactic guidance from interstitial scaffolds is now considered to be one of the important mechanisms for mammary cancer invasion (Gritsenko et al. 2012). The combined use of multiphoton and second-harmonic imaging shows that metastatic mammary cells migrating rapidly in vivo are closely associated with collagen-containing
fibers (Wang et al. 2002). PDAC is also a stroma-rich cancer. Instead of a mere bystander, PSCs together with the extensive ECM have been proposed to play a critical role in PDAC progression (Apte et al. 2012; Feig et al. 2012). Recent in vivo studies demonstrate that PSCs promoted not only the local tumor growth (Bachem et al. 2005), but more strikingly the metastasis of PDAC (Vonlaufen et al. 2008). Our data, indicating that PSC-SN stimulated the haptokinesis/haptotaxis of PCCs, unravels one of the possible mechanisms involved in pancreatic cancer metastasis. The implication of this mechanism is important when considering that PSC may accompany cancer cells during dissemination (Vonlaufen et al. 2008) and provide tumor-favorable substratum to support cell survival and migration.

4.2 Collagen I is the major component in PSC-SN that mediates the stimulatory effect on PCCs via integrin \(\alpha_2\beta_1\)

4.2.1 Collagen I is the major mediator in PSC-SN

Our in vitro data further clarified that collagen I as the major mediator for PSC-SN induced effects. This is proven by 3 points: 1) Collagen I is as effective as PSC-SN in promoting the adhesion, random motility and trans-migration of Panc1 and UlaPaCa. This stimulatory effect was not induced by other matrix proteins (fibronectin, laminin) secreted from PSCs. 2) In contrast to poly-L-lysine, a non-specific substratum, collagen I specifically stimulated the migration of PCCs besides its well-known effect on cell adhesion. PCCs stimulated by PSC-SN and collagen I showed resembling motile phenotype, which is characterized by a polarized morphology, i.e. a clear distinction between cell front and rear (Lauffenburger and Horwitz 1996). 3) Blocking antibodies against integrin \(\alpha_2\beta_1\), the primary receptor for collagen I on Panc1 and UlaPaCa cells, significantly attenuated the stimulatory effects of PSC-SN. However, the RGD peptide, as a competitive inhibitor for fibronectin, did not affect PSC-SN-induced cell adhesion or migration.

Panc1 is a cell line originating from primary pancreatic tumor, whereas UlaPaCa is isolated from the peritoneal metastasis. The two different cell lines showed distinct hapto-response to PSC-SN and collagen I. UlaPaCa trans-migration was tremendously accelerated only when there was a concentration gradient of pre-coated collagen I or PSC-SN, indicating haptotaxis is the major motile response of UlaPaCa. In Panc1 cells, however, a strong stimulation was observed even in the absence of gradient, suggesting haptokinesis is mainly responsible for the induced trans-migration.
4.2.2 Formation of fibril PSC-derived collagen I \textit{in vitro}

Fibrillar collagen (collagen I) is synthesized and injected into the endoplasmic reticulum as pro-\(\alpha\) chains (Fig. 30). After hydroxylation and glycosylation at certain residues, each pro-\(\alpha\) chain combines with two others to form procollagen, which is then taken into transport vesicles and secreted out. In the extracellular space, procollagen is cleaved by metalloproteinases and converted to collagen molecules (1.5 nm in diameter), which further self-assemble into ordered polymers called collagen fibrils (10-300 nm in diameter). Finally, these collagen fibrils aggregate into larger cablelike bundles which can be seen under the light microscope as collagen fibers (several micrometers in diameter) (Alberts et al. 1994).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fibril.png}
\caption{The intracellular and extracellular events in the formation of a collagen fibril. (A) Note that collagen fibrils are shown assembling in the extracellular space contained within a large infolding in the plasma membrane. As one example of how collagen fibrils can form ordered arrays in the extracellular space, they are shown further assembling into large collagen fibers, which are visible in the light microscope. The covalent cross-links that stabilize the extracellular assemblies are not shown. (B) Electron micrograph of a negatively stained collagen fibril reveals its typical striated appearance. This figure is cited from \textit{Molecular biology of the cell, 4\textsuperscript{th} edition, Part IV, Figure 19-47}.}
\end{figure}

According to the molecular biology of collagen, we speculate that there are procollagen I
and collagen I molecules in PSC-SN. During the in vitro incubation at 37°C in 5% CO2, the pH of the SN is adjusted to neutral and the temperature raises to physiological. Then the fibril formation of PSC-derived collagen will occur spontaneously resulting in banded fibers bound to the culture surface, consequently promoting migration of Panc1 and UlaPaCa. Further experiments are required to verify the biochemical characteristic of PSC-derived collagen I.

4.2.3 PSCs promote PCC migration by providing a pathway of collagen I

In other cancers with strong desmoplasia (mammary, prostate, lung cancer, etc.), collagen I is shown to be the key component of stroma in both primary and metastatic sites (Egeblad et al. 2010), and play a vital role in the development and progression of cancers (Cheng and Leung 2011; Cox et al. 2013; Li et al. 2010; Provenzano et al. 2008; Shintani et al. 2008; Sodek et al. 2008). In PDAC, collagen I is more frequently associated with epithelial-to-mesenchymal transition (EMT) (Imamichi et al. 2007; Koenig et al. 2006; Shintani et al. 2006), which is proposed to be a critical mechanism for the acquisition of malignant phenotypes by epithelial cancer cells (Thiery 2002). However, EMT is a time-consuming cellular process which consists of multiple biochemical events, such as activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, and changes in the expression of specific microRNAs (Kalluri and Weinberg 2009). Our study suggests that short time after stimulation with collagen I or PSC-SN (less than 1 hour), the cell adhesion, spreading and migration were already significantly initiated, indicating a distinct mechanism, rather than EMT, involved in the pro-migratory effect of collagen I and PSC-SN.

Grzesiak etc. (Grzesiak and Bouvet 2006) also showed in 8 pancreatic cell lines that collagen I promoted the strongest adhesion, proliferation, and migration compared with other substrates tested. Consistent with their data, we further indicate collagen I as a major component responsible for PSC-induced adhesion and migration of PCCs. This is more relevant to the in vivo environment where PSCs are in close proximity to PCCs and promote tumor progress via a paracrine pathway. Actually, the locomotive activation elicited by collagen I reflects a primary function of PSCs — to produce a scaffold that promotes cell movement. Thus it is plausible that through de novo synthesis and deposition of collagen I, PSCs accompany and favor PCC metastasis by providing trails of least resistance for cells to adhere and migrate.
4.2.4 PSC-derived collagen I initiated the integrin α2β1 pathway

ECM proteins induce intracellular signals in large part through integrin receptors (Hynes 1992). Together with ECM modification in PDAC, the expression of integrin profile is modulated in PCCs. The expression of α1, α2, α3, and α6 subunits is up-regulated in vivo; whereas α2, α3, α5, α6, αv and β1 are expressed on most pancreatic cancer cell lines (Grzesiak et al. 2007). Besides, expressions of integrin α6β1 (Vogelmann et al. 1999; Weinel et al. 1995) and αvβ3 (Hosotani et al. 2002) in pancreatic cancer cell lines and tissues are associated with invasion.

In our study, integrin α2β1 is identified as the motility-promoting receptor for haptokinesis of Panc1 and haptotaxis of UlaPaCa. Utilization of blocking antibody against integrin α2β1 significantly attenuated the stimulatory effect of PSC-SN and collagen I. Integrin-targeted therapy has revealed promising results in both preclinical and clinical studies in breast cancer, melanoma, glioblastoma and other solid tumors (for review see Desgrosellier and Cheresh 2010). In PDAC, inhibition of β1 integrin with monoclonal antibody strongly blocked cancer cell migration and invasion in vitro (Arao et al. 2000; Ryschich et al. 2009). In vivo, knockdown of β1 integrin reduced primary tumor growth by 50% and completely inhibited spontaneously occurring metastasis (Grzesiak et al. 2011). Consistently, our data provide additional insights to the combined blockage of integrin α2 and β1 subunits as a potential intervention in PDAC.

4.3 FAK signaling pathway is involved in PSC-derived, collagen I-induced haptokinesis and haptotaxis of PCCs

Integrin signaling functions are mediated by a variety of intracellular proteins which are associated with integrin cytoplasmic domains (Liu et al. 2000). FAK is one of the crucial proteins to transduce signals initiated by ECM-integrin interactions (Schlaepfer et al. 2004). Besides, recent studies delineate FAK as mechanosensor for the regulation of cell responses to ECM stiffness (Alexander et al. 2008; Plotnikov et al. 2012). Our data further reveal that FAK signaling pathway plays a vital role in the haptokinetic and haptotactic response of PCCs. In the time-course western blot, PSC-SN and collagen I induced a constantly enhanced phosphorylation of pFAK at Tyr397 in Panc1 and UlaPaCa. Inhibition of FAK, either by the phosphorylation inhibitor PF-573228 or by siRNA-mediated gene silencing, significantly attenuated PSC-SN and collagen I promoted haptokinesis of Panc1 and haptotaxis of UlaPaCa. FAK is increasingly accepted as a potential anti-cancer target
Both in vivo and in vitro studies suggest that inhibition of FAK resulted in decreased growth, metastasis and chemoresistance of PDAC (Duxbury et al. 2004; Hochwald et al. 2009; Huanwen et al. 2009; Stokes et al. 2011; Ucar et al. 2011). Moreover, a recent phase I trial of a FAK inhibitor in advanced solid tumors confirms its clinical safety and supports further investigation in cancer therapy (Infante et al. 2012). Our study demonstrated the involvement of FAK in PSC-induced PCC migration, which reinforces FAK as a promising target for PDAC therapy.

In summary, we demonstrate here that PSCs promote the migration of PCCs mainly via the haptokinetic and haptotactic mechanisms. Collagen I secreted from PSCs, in large part, mediates the hapto-migration of PCCs by enhancing integrin α2β1-FAK signaling pathway. Considering the interaction between PSCs and PCCs in vivo, our data present a novel view on the highly motile and early metastatic behavior of cancer cells, and suggest that integrin α2β1 and FAK are potential targets for preventing PDAC progression.
5. SUMMARY

Background, Hypothesis, and Aims: Pancreatic stellate cells (PSCs) are the key fibrogenic cells responsible for the strong desmoplasia in pancreatic ductal adenocarcinoma (PDAC). Recent studies show that PSCs promote metastasis as well as local growth of pancreatic cancer, but the underlying mechanisms are not clearly identified. Therefore, this study aimed to answer the following questions:

- Do PSCs stimulate trans-migration of pancreatic cancer cells (PCCs) in the modified Boyden chamber assay?
- Which biological steps involved in PCC trans-migration (e.g. adhesion, motility, directional migration) is/are affected by PSCs?
- Which factor produced by PSCs is mainly responsible for PSC-induced PCC trans-migration?
- Which intracellular signaling is initiated upon PSC-induced PCC trans-migration?

Methods: Human PSCs were isolated from cancerous pancreas by outgrowth method. The effect of PSC supernatant (PSC-SN) on the trans-migration, adhesion and motility of PCCs (Panc1, UlaPaCa) was investigated by modified Boyden chamber assay, adhesion assay and single cell tracking assay, respectively. The effects of collagen I, fibronectin, laminin or poly-L-lysine on PCCs were evaluated by coating the culture surfaces with each adhesive molecule. Organization of cytoskeleton and formation of focal adhesions (FAs) were examined by fluorescence stainings of F-actin, paxillin and phospho-focal adhesion kinase (FAK, Tyr397). Integrin expression and FAK phosphorylation were assessed by Western blot. Anti-integrin α2/β1 antibodies were used to verify the involvement of collagen I in PSC-SN-induced PCC migration. FAK inhibitor PF-573228 and siRNA-mediated FAK knockdown were applied to clarify the intracellular signaling pathway.

Results: PSC-SN dose-dependently induced trans-migration of Panc1 and UlaPaCa cells, mainly by improving cell adhesion and random motility. PSC-SN-mediated cell adhesion was a prerequisite for the stimulation on PCC migration. The chemokines contained in PSC-SN, however, were not sufficient to promote the migration of PCCs.

In contrast to poly-L-lysine, fibronectin and laminin, only collagen I showed comparable effect to PSC-SN on PCC behavior, including polarized morphology, facilitated adhesion,
SUMMARY

Accelerated motility, and stimulated trans-migration. Both PSC-SN and collagen I induced haptokinesis of Panc1 and haptotaxis of UlaPaCa cells. Blocking antibodies against integrin α2/β1 subunits significantly attenuated PSC-SN- and collagen I-induced PCC trans-migration as well as adhesion.

In PCCs stimulated by PSC-SN or collagen I, polymerization of F-actin and formation of focal adhesions were obviously induced. In a time course Western blot analysis, phosphorylation of FAK (Try397) was constantly enhanced by PSC-SN or collagen I. Inhibition of FAK function significantly diminished the effect of PSC-SN and collagen I on PCC hapto-migration.

Conclusions: Based on these findings we demonstrate, that

- PSC-SN promotes the trans-migration of Panc1 and UlaPaCa cells, mainly via its haptokinetic and haptotactic effect.
- Collagen I is the major mediator for PSC-SN-induced haptokinesis of Panc1 and haptotaxis of UlaPaCa cells.
- PSC-SN-induced PCC hapto-migration is largely dependent on the initiation of integrin α2β1-FAK signaling pathway.

Activated PSCs synthesize multiple cytokines and growth factors, which however fail to induce chemotaxis of Panc1 and UlaPaCa cells in our system. Instead, we clarify for the first time that PSCs promote the hapto-migration of cancer cells via integrin α2β1-FAK signaling pathway. This presents a novel view on the highly motile and early metastatic behavior of cancer cells, and implies that the integrin/FAK-based therapy might be efficient in inhibiting pancreatic cancer cell motility and metastasis.
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cancer stimulates COX-2 in PSCs. Mol Cancer 4: 27 (2005)
ACKNOWLEDGMENTS

7. ACKNOWLEDGEMENTS

It would never have been possible to accomplish this research project and doctoral thesis without the help and support of the kind people around me. I would like to express my thanks to all of them and extend my appreciation especially to the following.

Foremost, I would like to express my deepest gratitude to my principle supervisor Prof. Dr. Max G Bachem for giving me the opportunity to join his brilliant and friendly research group, for his continuous support of my Ph.D. research, his inspiring guidance and constructive advice. I also thank him for encouraging me to attend several academic conferences so that my scientific vision has been broadened and my communication skills have been further improved.

I must send my wholehearted thanks to my second supervisor Dr. Shaoxia Zhou. I thank her for the systemic guidance and great effort she put in training me, for all the genuine and pertinent instructions, her patience and continuous encouragement and motivation. Owing to the good advice, support and friendship of Dr. Zhou, I have learned not only how to be a scientist in the academic field, but also how to be a well-round person in the world.

I appreciate the Chinese Scholarship Committee for providing the financial support during my three years’ study in Germany. I also thank the GRK1041 for giving me the opportunity to join the excellent academic college, and supporting me to attend the Annual Meeting of the European Pancreatic Club in Switzerland.

I offer my respect and thanks to Prof. Dr. Thomas Seufferlein for his concerns in my experiments, his insightful comments and suggestions, and his generous help.

I am sincerely grateful to Dr. Hansjörg Habisch for being open to discussions and ideas, responding to my questions and queries so promptly, as well as the encouragement and friendship from him.

I would like to thank our technician Mrs. Gisela Sailer who is always kind and friendly, for giving me unreserved help in many different ways, for all the techniques I learned from her, and her expert technical assistance.

I appreciate the help from Prof. Dr. Karin Scharffetter-Kochanek and Dr. Dongsheng Jiang who kindly provided a melanoma cell line to meet our urgent need.

My thanks also go to Prof. Dr. Zilin Sun, who recommended me for the post in Prof.
Bachem’s department and encouraged me to start the Ph.D. career.

I thank all the staff in the Department of Clinical Chemistry for providing help whenever I was in need, making a friendly environment for working, as well as all the fun we enjoyed together.

My warm appreciation and friendship go to my dear friends: Fang Zou, Zhigong Zhang, Chunxiang Fan, Jinjing Ren, Bingquan Yang, Junwen Tan, Hang Li, Li Huang, Yan Wang, Shanhu Qiu, Zhi Zuo, with whom I shared my happiness and frustrations, and enjoyed the amazing and unforgettable life in Germany.

Last but not least, I am deeply grateful to my family, especially my parents who experienced the ups and downs of my research, believed in my decisions, and encouraged me throughout my life. I must thank Zheng Gong, my husband, for his continuous love, understanding and support.
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EDUCATION & TRAINING

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August 2003 - June 2008: Bachelor degree
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Project: Effect of self-monitoring of blood glucose or urine glucose on the management of patients with non-insulin-treated diabetes.
Skills: clinical trial design, management of Case Report Forms, follow-up of subjects, teamwork, negotiation, interpersonal skills

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Project: Epidemiological investigation of prenatal screening for Down's syndrome in parts of Nanjing. *(Executed Student Research Training Program funded by Southeast University)*
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**The 18th International Charles Heidelberger Symposium on Cancer Research**
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**The 6th Mildred Scheel Cancer Conference**
Gästehaus Petersberg, Königswinter near Bonn, Germany, June 5-7, 2013
Poster presentation: Pancreatic stellate cells promote hapto-migration of pancreatic cancer cells through collagen I-mediated activation of the α2β1 integrin pathway

**The 45th Annual Meeting of the European Pancreatic Club**
Zurich, Switzerland, June 26-29, 2013
Oral presentation: Pancreatic stellate cells promote hapto-migration of pancreatic cancer cells through collagen I-mediated activation of the α2β1 integrin pathway

**PUBLICATIONS**


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