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Impact by pancreatic stellate cells on EMT and pancreatic cancer cell invasion: adding a third dimension *in vitro*

Emelie Karnevi^{1,2*}, Ann H Rosendahl², Katarzyna Said Hilmersson¹, Moin A Saleem³, Roland Andersson¹

1. Lund University and Skåne University Hospital, Department of Clinical Sciences Lund, Division of Surgery, Lund, Sweden

2. Lund University and Skåne University Hospital, Department of Clinical Sciences Lund, Division of Oncology and Pathology, Lund, Sweden

3. University of Bristol, School of Clinical Sciences, Children's Renal Unit and Academic Renal Unit, Bristol, UK

*Corresponding author: Emelie Karnevi

Email: emelie.karnevi@med.lu.se

Running title

Pancreatic cancer invasion and EMT

Key words

Pancreatic cancer, pancreatic stellate cells, macrophages, epithelial-mesenchymal transition, invasion, 3D organotypic model

Summary statement (15-30 words)

The 3D organotypic model enabled the study of the complex pancreatic tumour microenvironment *in vitro*, where the pro-tumour influence by two of the major non-malignant cell types were visualised.

Abbreviations

ECM	Extra-cellular matrix
EMT	Epithelial-mesenchymal transition
TAM	Tumour-associated macrophage
TGF- β	Transforming growth factor β
TPSC	Tumour-associated pancreatic stellate cell
ZEB1	Zinc finger E-box binding homeobox 1
ZO-1	Zona occludens protein 1 / tight junction protein 1

Abstract

Pancreatic cancer is associated with a highly abundant stroma and low-grade inflammation. In the local tumour microenvironment, elevated glucose levels, the presence of tumour-associated stellate cells and macrophages are hypothesized to promote the tumour progression and invasion. The present study investigated the influence by the microenvironment on pancreatic cancer cell invasion *in vitro*. After co-culture with tumour-associated pancreatic stellate cells (TPSCs), pancreatic cancer cells displayed up to 8-fold reduction in levels of epithelial-mesenchymal transition (EMT) markers E-cadherin and ZO-1, while β -catenin and vimentin levels were increased. A 3D organotypic model showed that TPSCs stimulated pancreatic cancer cell invasion, both as single cell (PANC-1) and cohort (MIAPaCa-2) invasion. The combined presence of TPSCs and M2-like macrophages induced invasion of the non-invasive BxPC-3 cells. High glucose conditions further enhanced changes in EMT markers as well as the cancer cell invasion. In summary, co-culture with TPSCs induced molecular changes associated with EMT in pancreatic cancer cells, regardless of differentiation status, and the organotypic model demonstrated the influence of microenvironmental factors, such as glucose, stellate cells and macrophages, on pancreatic cancer cell invasion.

Introduction

Pancreatic cancer is characterised by a dense desmoplastic stroma, which may constitute the majority of the tumour mass. The stroma creates a tumour permissive niche and supports tumour progression, local invasion, metastasis and act as a treatment barrier (Whatcott et al., 2015, Erkan et al., 2012, Jaster and Emmrich, 2008). It is now widely accepted that the pancreatic stellate cells, myofibroblast-like cells in the pancreatic microenvironment, are responsible for this stromal reaction due to their ability to produce large quantities of ECM and influence the composition and turnover of ECM proteins (Yen et al., 2002, Erkan et al., 2009, Apte et al., 2004). In addition, they may promote the pathogenesis of pancreatic cancer by creating a favourable tumour microenvironment through secretion of growth- and angiogenic factors as well as stimulating cancer cell migration (Tod et al., 2013, Hwang et al., 2008, Masamune et al., 2008). Although pancreatic stellate cells have an established role in the desmoplastic stroma, further investigations are needed to elucidate the influence by paracrine communication and cell-cell interactions with the tumour microenvironment on invasive properties of the cancer cells.

In order for cancer cells to disseminate and metastasise, the epithelial phenotype and cell-cell adhesions are down-regulated in favour of a more mesenchymal-like phenotype. This process of epithelial-mesenchymal transition (EMT) has been acknowledged as an intrinsic part of cancer progression (Castellanos et al., 2013, Hotz et al., 2007, Nakajima et al., 2004). The most significant events are down-regulation of E-cadherin, upregulation of transcription factors slug and snail, and induced expression of vimentin and N-cadherin. With the complex microenvironment around this highly invasive tumour type, further understanding regarding

the local interplay between present cell types and soluble factors is needed to elucidate key mechanisms and interactions for potential future targets..

Pancreatic cancer is associated with low grade inflammation and one of the major risk factors for pancreatic cancer is chronic pancreatitis (Greer and Whitcomb, 2009, Lowenfels et al., 1993). The local inflammation involves a diverse leukocyte population of which a subtype of macrophages, commonly classified as tumour-associated macrophages (TAMs) or M2-like macrophages, is a major component. Macrophages are generally divided into two subtypes: classically activated inflammatory M1 macrophages and alternatively activated M2 macrophages (Murray and Wynn, 2011, Martinez et al., 2008). However, due to their plasticity, it is now recognised that macrophages adjust to each unique local microenvironment and the M1/M2 paradigm are merely ends of a phenotype spectrum (Ruffell et al., 2012, Qian and Pollard, 2010). TAMs are frequently found at the tumour periphery where they supply matrix remodelling enzymes and stimulate local invasion. TAMs may also play a role in EMT to facilitate dissemination of tumour cells, and in regulating the local inflammation to block anti-tumour responses (Neyen et al., 2013, Green et al., 2009, Wormann et al., 2014).

In addition to inflammation, pancreatic cancer is commonly linked to type 2 diabetes (Chari et al., 2008, Pannala et al., 2008). Diabetes and obesity have both been recognised among the top modifiable risk factors for pancreatic cancer (Bao et al., 2011, Huxley et al., 2005). At the time of diagnosis, up to 80% of cancer patients demonstrate reduced glucose tolerance or new onset type 2 diabetes, with elevated blood glucose levels as a consequence (Pannala et al., 2008, Permert et al., 1993). Hyperglycemia may both fuel tumour cell proliferation as well as

impair response to treatment (Karnevi et al., 2013). However, its role in tumour cell invasiveness remains poorly understood.

The complex interplay within the tumour microenvironment has been difficult to study through conventional 2D models *in vitro*. In the present study, a 3D organotypic model was established to mimic the complex tumour microenvironment and cellular interactions present, with the aim to investigate the influence by pancreatic stellate cells, M2-like macrophages and hyperglycemia on pancreatic cancer cell invasion. In addition, co-culture studies were performed to study the influence by paracrine signalling on EMT in pancreatic cancer cells.

Material and methods

Materials

All materials and reagents were purchased from Sigma-Aldrich unless stated otherwise (St Louis, MO, USA). Cell culture medium, fetal bovine serum, penicillin-streptomycin, insulin-transferrin-selenium (ITS) and L-glutamine were obtained from Invitrogen (Paisley, UK). Antibody specifications, concentrations and suppliers are listed in Table 1.

Cell culture

The moderately differentiated BxPC-3, and poorly differentiated PANC-1 and MIAPaCa-2 human pancreatic cancer cell lines were purchased from ATCC-LGC Standards (Manassas, VA, USA). The cells were maintained in RPMI1640 or DMEM medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a humidified 5% CO₂ atmosphere at 37°C. The monocytic cell line THP-1 was purchased from ATCC and maintained in RPMI1640 medium supplemented with 10% FBS, 0.1% 2-mercaptoethanol and antibiotics as above. The tumour-derived pancreatic stellate cells (TPSCs) are conditionally immortalised human stellate cells generated from a pancreatic ductal adenocarcinoma specimen as previously described (Rosendahl et al., 2015). Briefly, cells were isolated by the outgrowth method and transformed with a temperature-sensitive SV40 large T-antigen and human telomerase. The TPSCs were maintained at 33°C where proliferation was sustained. Prior to experiments, cells were grown to 80% confluence and transferred to 37°C for seven days to inactivate the SV40LT and resume their primary phenotype. The cells were maintained in RPMI1640 medium supplemented with 10% FBS, 1% ITS and antibiotics as above. All experiments were performed in glucose-free RPMI1640 or DMEM supplemented with 5 mM (normal) or 25 mM (high) D-glucose, 2 mM L-

glutamine, and antibiotics as above (normal or high glucose medium; NGM or HGM), unless stated otherwise.

M2-like macrophage differentiation

The THP-1 cells were seeded in T-25 flasks (7.5×10^5) in NGM or HGM supplemented with 10% FBS and left overnight. The cells were then incubated with 60 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24h, followed by 48h incubation with 60 ng/ml PMA, 20 ng/ml IL-4 and 20 ng/ml IL-13. M2-like macrophage differentiation was verified morphologically under microscope and by multicolour flow cytometry (CD11c (clone B-ly6), CD14 (clone M5E2), CD206 (clone 19.2) and HLA-DR (clone G46-6); BD Biosciences, San Diego, CA, USA) using a FACSCalibur (BD Biosciences) dual laser flow cytometer with Cell Quest Pro software (BD Biosciences) and analysed using FlowJo software.

Co-culture

The PANC-1, MIAPaCa-2 and BxPC-3 cell lines were seeded in 6-wells plates (4×10^5 cells/well) and co-cultured for 48h with or without TPSCs or M2-like macrophages seeded in inserts (3 μ m pores; 8×10^5 cells/insert). Alternatively, pancreatic cancer cells were incubated with or without 5 ng/ml TGF- β for 48h. The cancer cells were then harvested and transferred to inserts (8 μ m pores) with NGM or HGM supplemented with 10% FBS in the lower chamber. After 6h incubation, cancer cell lysates were collected and stored at -80°C .

Western immunoblotting

Samples were prepared and Western immunoblotting performed as outlined previously (Karnevi et al., 2013). Membranes were incubated overnight at 4°C with anti-human slug, claudin-1, ZO-1, ZEB-1, E-cadherin, vimentin or β -catenin primary antibodies. β -actin

(BxPC-3) or GAPDH (PANC-1 and MIAPaCa-2) were used as loading controls. The following day membranes were washed, incubated with HRP-conjugated secondary antibody for 1h and visualised using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, Waltham, MA, USA). Immunoblotted proteins were detected using LI-COR Odyssey system with ImageStudio software (LI-COR Biosciences, Ltd., Cambridge, UK) and quantified using ImageJ software (NIH, USA).

3D organotypic assay

The three-dimensional (3D) organotypic model was set up as described by Moutasim *et al* and Froeling *et al* (Moutasim et al., 2011, Froeling et al., 2010). To make the gel, a mixture of 3.5 parts Collagen I (Millipore, Temecula, CA, USA), 3.5 parts Matrigel (Corning, Tewksbury, MA, USA), 1 part DMEM 10X, 1 part FBS and 1 part cell suspension was added to 24-well plates (1 ml/well) and incubated for 1h at 37°C. The cell suspension contained 2.5×10^5 TPSCs with or without 2.5×10^5 M2-like differentiated macrophages in NGM or HGM supplemented with 10% FBS. After the gels had polymerised, medium was added on top and the gels left overnight at 37°C. Cancer cells (5×10^5) mixed with TPSCs (2.5×10^5) were then seeded on top of the gels and incubated 24h. On day three, the gels were raised by inserting metal grids in 6-wells plates with collagen-coated nylon sheets placed on top. The gels were transferred to the nylon-sheets and medium added to the well up to the bottom of the gel to create an air-liquid interface. The medium was changed every 2-3 days. After 7 or 14 days incubation, the gels were bisected, formalin fixed and paraffin embedded.

Histological and immunohistochemical analyses

Staining procedures

Paraffin-embedded organotypic gels were sectioned 5 μm thick, deparaffinised and rehydrated. For histological evaluation, sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome according to the manufacturer's instructions.

Prior to immunohistochemical stainings, sections were subjected to antigen retrieval, either using citrate buffer (pH 6) or Tris-EDTA buffer (pH 9), for 10 min at 90°C in waterbath. The organotypic sections were subsequently blocked with 5% horse serum, avidin/biotin and incubated with anti-human Ki-67 or cytokeratin primary antibodies overnight at 4°C. The following day, sections were incubated with HRP-conjugated secondary antibody for 1h, and visualised using Vectastain Elite ABC kit and DAB (Vector, Peterborough, UK).

Prior to immunofluorescence staining, antigen retrieval was performed using citrate buffer as above. The sections were subsequently blocked with 5% donkey serum, and incubated with anti-human β -catenin, E-cadherin, vimentin or ZO-1 primary antibodies overnight at 4°C. The next day, sections were incubated with Alexa Fluor® 555-conjugated secondary antibody for 1h. The sections were washed and nuclei counterstained with DAPI.

Quantification

The organotypic sections were photographed at 20X magnification with CellSens Dimension software. Whole slide digital images were captured at 40X magnification using Aperio ScanScope slide scanner (Aperio Technologies, Leica Biosystems, Nussloch, Germany). Total gel width, thickness of tumour epithelium and distance covered by invading cells were analysed with ImageScope software (Leica Biosystems).

Statistical analyses

Western immunoblot data were analyzed by two-way ANOVA with Bonferroni *post-hoc* test using GraphPad prism software. All graphs are expressed as mean \pm SE of a minimum of three individual experiments. Statistical analyses of organotypic sections were performed

using unpaired two-tailed Student's *t*-test using Excel 2010 software. Graphs represent mean \pm SE of six measurements of depth of invasion in relation to total gel width. A *P*-value of <0.05 was considered statistically significant.

Results

TPSCs induced changes associated with EMT in pancreatic cancer cells.

To investigate the impact of paracrine signalling by stellate cells on EMT, the BxPC-3, PANC-1 and MIAPaCa-2 pancreatic cancer cells were cultured in the presence of TPSCs without direct cell contact. The moderately differentiated BxPC-3 cells displayed basal expression of E-cadherin and the tight junction protein ZO-1, both of which decreased by 15% ($P<0.05$) and 4-fold ($P<0.001$), respectively, in response to TPSCs (Fig. 1A). In addition, TPSCs stimulated a 2-fold increased expression of the transcription factor slug ($P<0.05$). Similarly, the poorly differentiated PANC-1 cells displayed 8-fold ($P<0.001$) and 2-fold ($P<0.05$) reduced levels of E-cadherin and ZO-1, respectively, after TPSC co-culture (Fig. 1A), of which ZO-1 was further reduced in the presence of high glucose levels ($P<0.05$). PANC-1 also showed almost 2-fold increased levels of the mesenchymal marker vimentin following co-culture ($P<0.001$). Lastly, MIAPaCa-2, the poorly differentiated and most mesenchymal and spindle-like cells in culture, lacked basal E-cadherin expression but demonstrated strong expression of vimentin, which was further increased by TPSC-derived factors ($P<0.01$; Fig 1A). In addition, elevated ZO-1 ($P<0.01$) and β -catenin ($P<0.05$) levels were observed following TPSC co-culture, and more pronounced under high glucose conditions.

The TPSC-induced EMT changes were similar to or greater than those by the known EMT-inducer TGF- β (Fig. 1A-B). These results demonstrated that soluble factors and paracrine signalling by TPSCs induced molecular changes associated with EMT in pancreatic cancer cells of diverse differentiation status.

TPSCs enhanced invasion by PANC-1 and MIAPaCa-2 cells.

In order to mimic the tumour microenvironment and investigate the influence by cell-cell communication between pancreatic cancer cells and TPSCs on cancer cell invasion, a 3D organotypic model was established. No signs of invasion was observed for BxPC-3 cells after 7 or 14 days culture, regardless of the presence of pancreatic stellate cells or glucose levels (Fig 2A, C). In contrast, PANC-1 and MIAPaCa-2 cells displayed induced invasion at day 7 in the presence of TPSCs, which was 28-31% ($P<0.01$) and 11-14% ($P<0.05$) higher than compared with control gels without TPSCs, respectively (Fig. 2A, C). The invasion was further increased with 20-40% by day 14 ($P<0.01$), and under higher glucose levels. On average, the cell lines' invasive front was seen at the depth of 58% of the total gel width, compared with 43% at day 7. The two cell lines displayed different manners of invasion, where PANC-1 cells invaded the gel as single cells or small cell clusters (<5 cells) and MIAPaCa-2 displayed cohort invasion. The invasion was further verified by Trichrome staining of collagen fibres between invading cells and the tumour cell epithelium (Fig 2B), and cytokeratin staining of epithelial cells (Fig. S1).

Invading cell types displayed high levels of vimentin and lacked E-cadherin.

Associations between invasion pattern and EMT was further studied and the organotypic gel sections in the presence of TPSCs were evaluated for cellular localization of the EMT markers E-cadherin, β -catenin, vimentin and ZO-1. No changes in EMT marker levels were observed between days 7 and 14. The non-invasive and vimentin negative BxPC-3 cells displayed membranous E-cadherin and β -catenin expression throughout the tumour epithelium, with a weak membranous ZO-1 expression at the leading edge (data not shown). PANC-1 cells in contrast, demonstrated distinct vimentin expression in both the tumour epithelium and in invading cells (Fig. 3). The vimentin intensity was more pronounced in high

glucose conditions. A diffuse cytosolic E-cadherin and ZO-1 expression, and sparse membranous β -catenin expression was observed in a limited number of cells (Fig. 3). Similarly, the vimentin-positive MIAPaCa-2 tumour epithelium lacked E-cadherin expression but displayed sparse β -catenin and diffuse cytosolic ZO-1, with stronger intensity at the invasive front (data not shown).

M2-like macrophages facilitated invasion of the epithelial-like BxPC-3 pancreatic cancer cells.

The joint effects of TPSCs and M2-like macrophages in the microenvironment on tumour cell invasion were investigated next. After 14 days organotypic cultures in the presence of both M2-like macrophages and TPSCs, invasion of the previously non-invasive BxPC-3 cells was induced, compared with TPSC alone (Fig 4A; $P<0.05$). The BxPC-3 tumour epithelium made up for less than 20% of the total gel width, both with and without the presence of M2-like macrophages, while the induced invasion was displayed at the average depth of 40% of the total gel width. The BxPC-3 cells displayed characteristics of cohort invasion and the invasion was further enhanced by up to 40% in high glucose conditions ($P<0.05$). No significant difference in invasion was observed for PANC-1 and MIAPaCa-2 cells in the presence of M2-like macrophages in this model, compared with TPSCs alone (Fig 4B).

The presence of M2-like macrophages was associated with expression of mesenchymal markers in invading BxPC-3 cells.

To study if cell-cell interactions and the combined presence of macrophages and pancreatic stellate cells influenced EMT in invading cells, the cellular expression of selected EMT markers were evaluated. The presence of M2-like macrophages stimulated invasion with concomitant changes in EMT in invading BxPC-3 cell cohorts, but not in the tumour

epithelium, compared with TPSCs alone (Fig. 5). The BxPC-3 cells displayed loss of both E-cadherin and β -catenin in invading cell populations compared with the tumour cell epithelium (Fig. 5). In addition, a moderate expression of vimentin was induced in invading groups of BxPC-3 cells in the presence of M2-like macrophages. ZO-1 expression was observed at the leading edge in the tumour epithelium and was considerably enriched in invading cell cohorts (Fig. 5). The expression of the EMT markers were similar between low 5 mM and high 25 mM glucose levels. No differences in EMT markers were observed in PANC-1 and MIAPaCa-2 cells in the presence of M2-like macrophages compared with TPSCs alone (data not shown).

Macrophage-derived factors stimulate changes in EMT-associated markers

Finally, the contribution of paracrine signalling and soluble mediators by M2-like macrophages on tumour cell EMT was investigated through indirect co-culture between the two cell types. M2-like macrophages did not induce changes in the investigated EMT-markers to the same extent as TPSCs (Fig 6A). Macrophage-derived factors reduced the levels of slug in BxPC-3 ($P<0.05$), while no changes were seen for E-cadherin or ZO-1. PANC-1 however, displayed decreased E-cadherin levels by 25%, but also 2-fold reduced vimentin levels (both $P_s<0.01$). The reduction in vimentin was further enhanced by high glucose conditions ($P<0.05$). ZO-1 was increased in both PANC-1 and MIAPaCa-2 cells.

Discussion

The major non-malignant cellular components of the tumour microenvironment, the pancreatic stellate cells and tumour-associated macrophages, have been suggested to be involved in the progression of pancreatic cancer by creating a favourable tumour microenvironment and stimulate invasion, tumour growth, and development of metastases (Hwang et al., 2008, Masamune et al., 2008, Neyen et al., 2013, Green et al., 2009). The present study utilized the benefits of a 3D organotypic model to study the joint influence by pancreatic stellate cells and M2-like macrophages on the invasive characteristics of pancreatic cancer cells. Our results showed TPSC-derived factors to stimulate changes associated with EMT in pancreatic cancer cells of diverse differentiation status, and the combined presence of M2-like macrophages and TPSCs to induce invasion by the more epithelial-like BxPC-3 cells.

In this study, co-culture with tumour-associated pancreatic stellate cells (TPSCs) stimulated changes in EMT-associated proteins for all cell lines investigated. The cancer cells showed decreased E-cadherin levels, upregulation of vimentin as well as reduced levels of the tight junction protein ZO-1. These results are in line with previous studies where pancreatic stellate cells or cancer-associated fibroblasts have been shown to potentially induce EMT in pancreatic cancer cells, and thereby facilitate invasion (Kikuta et al., 2010, Froeling et al., 2009). Furthermore, the present study demonstrates TPSCs to induce EMT-associated changes by paracrine communication, regardless of differentiation status. It has been hypothesised that TPSCs potentially exert some of their tumour promoting effects through TGF- β , a known inducer of EMT. Here, the changes displayed after co-culture with TPSCs were similar to the effect of TGF- β treatment. This indicates that TGF- β may at least in part

contribute to the TPSC-induced changes in EMT observed. However other factors secreted by TPSCs such as uPA, FGF and PDGF may likely also be involved (Rosendahl et al., 2015).

It has been shown that cancer-associated fibroblasts or TPSCs can stimulate migration or invasion of cancer cells *in vitro*, although mainly through 2D models (Hwang et al., 2008, Kikuta et al., 2010). The organotypic model has previously displayed favourable results for visualisation of cancer cell invasion and interplay with the local microenvironment (Froeling et al., 2009, Linde et al., 2012). The results here demonstrate cell-cell interactions with TPSCs to stimulate different manners of invasion: single cell invasion (PANC-1) and cohort invasion (MIAPaCa-2). The pancreatic cancer cells also displayed significantly increased invasion in the presence of TPSCs, compared with gels lacking stellate cells. The epithelial-like BxPC-3 cells, on the other hand, remained non-invasive with an intact tumour epithelium. The cell lines' invasive behaviour displayed in the organotypic model corresponds to changes in EMT markers visualised by Western blot. This strengthens the pro-tumour role of TPSCs in the tumour microenvironment, and the evidence of paracrine communication and cell-cell interactions with pancreatic cancer cells. Furthermore, it suggests TPSCs to be an important target with the potential to regulate invasion and the associated EMT. Previous studies have focused on other pro-tumour properties of stellate cells in order to inhibit the tumourigenesis. The desmoplastic stroma produced by pancreatic stellate cells has been shown to create a treatment barrier around the tumour, limiting bioavailability of drugs (Jaster and Emmrich, 2008, Erkan et al., 2012, Whatcott et al., 2015). To surpass this barrier, Olive *et al.* increased drug availability and chemotherapy response by targeting the pancreatic stellate cells and thereby reducing the stromal compartment before administrating treatment (Olive et al., 2009). There has however been other studies demonstrating anti-tumour influences by the stroma, whereby deleting the stromal compartment results in increased aggressiveness of

pancreatic tumours and reduced survival (Rhim et al., 2014, Ozdemir et al., 2014). Based on the conflicting results by targeting the stroma, future investigative studies to reduce tumour aggressiveness and/or inhibit tumour progression stimulated by pancreatic stellate cells will require careful design in order to solely target the pro-tumour properties.

In the local pancreatic tumour microenvironment, tumour-associated macrophages (TAMs) play a central role next to the pancreatic stellate cells. In a study with squamous carcinoma cells, M2 macrophages together with fibroblasts enhance the invasion rate of cancer cells compared to fibroblasts alone (Linde et al., 2012). In a similar manner, the combined presence of TPSCs and M2-like macrophages in the present study induced invasion of more epithelial-like BxPC-3 pancreatic cancer cells, which displayed no invasion in the presence of TPSCs alone. Interestingly, the BxPC-3 cells disrupted the previously intact tumour epithelium and displayed invasion with an associated loss of E-cadherin expression. Staining of EMT markers displayed expression patterns resembling the more poorly differentiated PANC-1 and MIAPaCa-2 cells, in particular in the invaded groups of BxPC-3 cells. When investigating the influence on EMT-associated markers after co-culture, the M2-like macrophages induced changes that differed from those demonstrated by TPSCs-derived factors, such as reduced vimentin levels and increased ZO-1. This may be due to differences in secreted factors and thereby the paracrine signalling pathways involved. The M2-like macrophages most likely stimulate invasion and changes in EMT-markers by secretion of cytokines, MMPs and growth factors, which all are associated with invasion, metastasis and ECM degradation (Goswami et al., 2005, Baran et al., 2009, Kessenbrock et al., 2010, Kuwada et al., 2003, Lesina et al., 2011). In studies investigating colon cancer and breast cancer, a chemotactic loop between cancer cells and macrophages have been observed where the macrophages stimulate cancer cell invasion by secretion of EGF (Green et al., 2009, Goswami et al., 2005). The interplay

between TPSCs and macrophages themselves is also of interest, since pancreatic stellate cells have been suggested to contribute to local inflammation and recruitment of inflammatory cells (Tjomsland et al., 2011, Erez et al., 2010). Erez *et al* investigated inflammatory profiles of cancer-associated fibroblasts in a wide range of cancers, and found similar signatures comparing pancreatic cancer to the inflammatory environments present in skin cancer (Erez et al., 2010).

In conclusion, TPSCs induced changes associated with EMT in human pancreatic cancer cells and stimulated both single cell invasion (PANC-1) and cohort invasion (MIAPaCa-2). This suggests the inter-cell communication with pancreatic stellate cells to be central for pancreatic cancer cell invasion, and an important potential target to restrict tumour spreading. The presence of M2-like macrophages stimulated the invasion further as well as induced invasion of more epithelial-like and non-invasive cells. This demonstrates the importance of the tumour microenvironment in stimulating cancer progression and cancer cell invasion into surrounding tissue and opens up for further investigations in the search of new targets outside the tumour itself in order to inhibit the tumour progression.

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Competing interests

No potential conflicts of interest were disclosed.

Author contributions

EK designed the study. EK, KS, AHR and MAS made significant contributions to the experimental design, acquisition and interpretation of data, manuscript preparation and editing. RA provided funding for the study and manuscript editing. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Modulation of EMT markers in three pancreatic cancer cell lines

Representative images of Western immunoblotting of claudin-1, slug, vimentin, β -catenin, E-cadherin, ZO-1 and ZEB1 in cell lysates of BxPC-3, PANC-1 and MIAPaCa-2 after 48h co-culture with TPSCs (A) or 48h exposure to 5 ng/ml TGF- β (B) under normal (5 mM) or high (25 mM) glucose levels. β -actin (BxPC-3) or GAPDH (PANC-1, MIAPaCa-2) were used as loading controls. Graphs represent quantification of a minimum of three individual experiments with results presented as mean \pm SE, relative to control. Statistical analyses testing the significant difference between co-culture and control for both glucose levels were performed using two-way ANOVA with Bonferroni *post-hoc* test. N.D. = not detected

Figure 2. The invasive properties of pancreatic cancer cells using a 3D organotypic model

Histological analyses of gel sections from organotypic models of BxPC-3, PANC-1 or MIAPaCa-2 after 7 or 14 days culture in normal (5 mM) or high (25 mM) glucose conditions. A) H&E staining of organotypic models with or without TPSCs, B) Trichrome staining displaying collagen fibre in blue, C) graphs represent quantification of depth of invasion (% of total gel width) at day 7 or 14 of gels with TPSCs (empty bars) or without TPSCs (filled bars) as mean \pm SE. Representative images (20X magnification) from one of three independent experiments are shown. Scale bar = 50 μ m.

Figure 3. Distribution of EMT markers in tumour cell epithelium and invading cells in 3D organotypic model

Immunofluorescent staining of EMT markers vimentin, E-cadherin, β -catenin and ZO-1 in gel sections from organotypic model of moderately invasive PANC-1 after 14 days culture in normal (5 mM) or high (25 mM) glucose. Nuclei were counterstained with DAPI. Representative images (20X magnification) from one of three independent experiments are shown. Scale bar = 50 μ m.

Figure 4. Influence by M2-like macrophages on pancreatic cancer cell invasion

H&E staining of gel sections from organotypic models of A) BxPC-3, B) PANC-1 or MIAPaCa-2 cells after 14 days culture in the presence of TPSCs, with or without M2-like macrophages, under normal (5 mM) or high (25 mM) glucose conditions. Graph display quantification of depth of invasion (% of total gel width) at day 7 and 14 of BxPC-3 gels with M2-like macrophages (empty bars) or TPSCs alone (filled bars) as mean \pm SE. Representative images (20X magnification) from one of three individual experiments are shown. Scale bar = 50 μ m.

Figure 5. Distribution of EMT markers in tumour cell epithelium and invasive cells in the presence of both TPSCs and M2-like macrophages

Immunofluorescent staining of EMT markers vimentin, E-cadherin, β -catenin and ZO-1 in gel sections from organotypic models of BxPC-3 pancreatic cancer cells in the presence of TPSCs, with or without M2-like macrophages, under normal (5 mM) or high (25 mM) glucose conditions. Nuclei were counterstained with DAPI. Representative (20X magnification) from one of three independent experiments is shown. Scale bar = 50 μ m.

Figure 6. Influence by macrophage-derived factors on EMT-associated markers

Representative images of Western immunoblotting of claudin-1, slug, vimentin, β -catenin, E-cadherin, ZO-1 and ZEB1 in cell lysates of BxPC-3, PANC-1 and MIAPaCa-2 after 48h co-culture with M2-like macrophages. β -actin (BxPC-3) or GAPDH (PANC-1 and MIAPaCa-2) were used as loading controls. Graphs represent quantification of a minimum of three independent experiments with data presented as mean \pm SE, relative to control. Statistical analyses testing the significant difference between co-culture and control for both glucose levels were performed using two-way ANOVA with Bonferroni *post-hoc* test. N.D. = not detected