Cancer Associated Fibroblasts Derived from Pancreatic Adenocarcinoma and Their Role in Cell Migration

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Abstract. *Background/Aim: Pancreatic ductal adenocarcinoma (PDAC) shows poor survival and early systemic dissemination. Cancer associated fibroblasts (CAFs) enhance migration and invasion of cancer cells. We aimed to investigate the role of CAFs in cell migration and their underlying paracrine effects. Materials and Methods: Using Transwell® migration assays, PDAC cells (PANC-1) and three distinct types of fibroblasts were analyzed: CAFs, genetically transformed human foreskin-fibroblasts (BJeLR), and non-transformed human foreskin-fibroblasts (VH7). IL6 in the culture supernatant was measured to investigate paracrine communication in monocultures and direct/indirect cocultures. Results: CAFs showed a significantly higher capacity to migrate in vitro when compared to benign fibroblasts ($p=0.009$). They also facilitated the migration of PDAC cells in coculture ($p=0.001$). Neither BJeLR, nor VH7 displayed such features. This was accompanied by a significant increase in IL-6 when CAFs were cocultured with PANC-1 ($p=0.009$). Conclusion: CAFs are a key element of intra-tumoral migration and should be further investigated as a potential therapeutic target.*

Patients with pancreatic ductal adenocarcinoma (PDAC) display poor 5-year survival rates of approximately 9%, concomitant with steadily increasing incidence rates (1). In addition, pancreatic carcinoma cells tend to metastasize at a very early stage (2) and when diagnosed, about 53% of the

patients with PDAC present with systemic dissemination (3). Early metastasis limits surgical treatment options and due to greatly ineffective chemotherapies, the patient's overall survival drops significantly (4). Therefore, underlying mechanisms that cause early metastasis are of major interest.

A dense and fibrotic desmoplastic stroma is characteristic of the PDAC. The pancreatic tumor microenvironment largely consists of inflammatory and immune cells, extracellular matrix and foremost cancer-associated fibroblasts (CAFs) (5, 6), which all play a significant role in tumor spread and therapy resistance (7-11). Each of the cell types have multiple functions, some of which help the pancreatic cancer cells sustain and enhance their malignant potential. Here, CAFs are of particular interest, exhibiting functions that enable and enhance the migration of cancer cells and promote a pro-invasive tumor environment (5, 12-15).

CAFs originate from various pancreatic cell types, such as pancreatic stellate cells, resident fibroblasts, bone marrow derived cells (BMDC) and epithelial cells (5). Even in the early stages of pancreatic cancer development/carcinogenesis, carcinoma cells establish a specific cancer microenvironment, which activates fibroblasts (16-19). Activated CAFs are characterized by unique expression of α -SMA, in addition to Vimentin and GFAP (11, 20).

CAFs are able to communicate with the cells in their direct tumor microenvironment by direct cell-cell contact as well as in a paracrine manner (21). Hereto, CAFs can secrete pro-inflammatory cytokines such as IL1 β , IL6, IL10, and TNF α (22, 23). In particular, IL6, which acts in the IL6/STAT3 pathway, can be regarded as a pivotal actor initiating and promoting carcinogenesis, while orchestrating the epithelial-mesenchymal transition and metastasis in pancreatic cancer (24-30). Moreover, increased IL6 serum levels in the clinical setting have been associated with liver metastasis, advanced stage cancer and limited survival (31).

Here, we focused on further investigating the underlying paracrine effects of primary pancreatic cancer fibroblasts on

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Table I. Specifications of the donors of patient derived cancer associated fibroblasts (CAFs). All CAFs were obtained from pancreatic adenocarcinoma specimens.

Patient	Age	Gender	Tumor type	T Stage	N Stage	Grade	Passages used for analysis
1	53	m	Adenocarcinoma	2	2	G3	2, 3, 5
2	57	w	Adenocarcinoma	2	1	G2	2, 3, 4
3	52	w	Adenocarcinoma	3b	1	G3	1, 2

migration as a key mechanism of metastasis in the PDAC microenvironment (32). We used a Transwell® migration assay in which pancreatic cancer cells (PANC-1) and fibroblasts were spatially separated but able to communicate in a paracrine manner.

In search of the molecular mechanisms by which CAFs potentially impact and somehow maintain their pro-cancerous effects during *in vitro* cultures, genetic alterations were also investigated (33, 34). Notably, down-regulation of p53 may distinguish CAFs from other fibroblasts (35). Additionally, conditioned medium from fibroblasts with down-regulated p53 expression was shown to increase tumor cell migration *in vitro* (36).

In order to investigate the pro-invasive effects of CAFs, we compared CAFs isolated directly from resected pancreatic cancer specimens with the genetically transformed human foreskin derived fibroblasts BJeLR (BJ fibroblasts transformed with hTERT, genomic SV40 LT and ST oncoproteins, and oncogenic HRASV12). These fibroblasts were immortalized *via* an hTERT vector by lentiviral transfection, but also equipped with the oncogenic SV40T and HRASV12, altogether suppressing the cell cycle control protein p53 (37). Non-transformed human foreskin fibroblasts (VH7) served as a non-malignant control.

Materials and Methods

Patient derived cell lines. Three patients who suffered from resectable PDAC and gave informed consent before operation served as tissue donors. Exclusion criteria were pregnancy and age under 18 years. Analyses were conducted following approval from the institutional review board (EA1/292/16) and in accordance with the Helsinki Declaration of 1975.

Cell culture. Three different types of fibroblasts; primary pancreatic CAFs, BJeLR, VH7 and a pancreatic cancer cell line (PANC-1) were used for the experiments. In addition, CAFs were derived from the tumor tissues of three individuals suffering from PDAC (Table I). Derivation protocols were described previously (38). In brief, the tissue was dissociated mechanically, rinsed with cell culture medium and seeded into cell culture flasks. CAFs were characterized using immunohistochemical staining following established protocols (39), which used vimentin-rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, CA, USA), monoclonal mouse anti-gial fibrillary

acidic protein (GFAP) antibody (Sigma Aldrich, St. Louis, MO, USA) and monoclonal mouse anti-actin α -smooth muscle (α -SMA) antibody (Sigma Aldrich). Primary fibroblast cultures were continued when mesenchymal origin with high purity had been validated. VH7 is a non-transformed human foreskin cell line that served as a non-malignant control, as previously described (40). BJeLR cells are human foreskin fibroblasts transformed with a vector containing the human telomerase reverse transcriptase (hTERT), genomic SV40 LT and ST oncoproteins and oncogenic HRASV12. They were obtained directly from Hahn *et al.* and kindly provided by Prof. Dr. rer. nat. Reinhold Schäfer (Charité Universitätsmedizin Berlin) (37). PANC-1 is a commercially available cell line of pancreatic adenocarcinoma. All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin and streptomycin and incubated at 37° in humidified air containing 5% CO₂.

For verification of the correct identity and origin of the cell lines, cell authentication was carried out by DNA fingerprinting, which used 8 different and highly polymorphic short tandem repeat (STR) loci. In addition, all samples were tested for the presence of mitochondrial DNA sequences from rodent cells. Analyses were carried out by Leibniz-Institute German Collection of Microorganisms and Cell Cultures.

Migration assay. To analyze the migration of cells, standardized cell migration assay was performed, using 8 µm pore Transwell® cell culture inserts (Falcon®, Cat #353097) in a 24-well plate. For the monoculture setup, cells were trypsinized and 10⁴ cells were seeded into the upper chamber of the Transwell® cell culture inserts in fetal bovine serum (FBS) free cell culture medium. The bottom chamber was filled with cell culture medium containing FBS. In an indirect co-culture setup, 5×10⁴ cells were additionally seeded into the lower chamber. After 24 h incubation, the Transwell® membranes were washed and stained with 0.05 % Crystal violet for 20 min.

The membrane was captured at 5× magnification by analyzing four visual fields per sample. The results were displayed as mean pixels/area and counted *via* Fiji using a macro for the threshold and pixel analysis function as described previously (41, 42).

ELISA. Cell culture supernatant was collected from a CAF and PANC-1 monoculture and co-culture set-up in a standardized fashion, as previously described (38). The levels of IL6 in the medium were measured using a Human IL-6 ELISA set (BD OptEIA™, San Jose, CA, USA, Cat# 555220). The ELISAs were performed according to the manufacturer's instructions and the levels of IL6 were normalized to the number of cells used in each culture.

Statistical analysis. Statistical analyses were performed with GraphPad Prism (version 8.4.0 for MacOS, GraphPad Software, San

Figure 1. Immunohistochemistry staining of one representative cell line of primary pancreatic cancer associated fibroblast (CAF), using α -SMA (A), Vimentin (B), GFAP (C). All patient-derived primary pancreatic cancer fibroblasts were characterized as CAFs for all sources.

Figure 2. Fibroblast migration: The migration of VH7 ($n=3$), BJELR ($n=3$) and primary cancer associated fibroblast (CAF) ($n=9$) was observed in a monoculture setup during a period of 24 h. All fibroblasts showed a capacity of migration. Primary pancreatic CAFs migrate significantly more than benign fibroblasts (VH7). $**p \leq 0.01$.

Diego, CA, USA). For the statistical analysis all data obtained from the migration assays and the cell culture supernatant analysis was tested without following the non-Gaussian distribution. To test more than two groups, the Kruskal-Wallis test was performed followed by a post-hoc Dunn's multiple comparisons test. When significance correlation was tested, results were followed by a Mann-Whitney *U*-test for testing independent variables in a pairwise comparison. For matching pairs, a Wilcoxon signed-rank test was conducted. In all tests performed, $p < 0.05$ characterized significant correlations.

Results

Characterization of CAFs. CAFs of three patients suffering from PDAC were isolated (Table I). Before conducting additional studies, we ensured that the isolated fibroblasts from the three individuals could be defined as CAFs, all co-expressing α -SMA, Vimentin and GFAP (Figure 1).

CAF_s show significant capacity for migration. To study the migration capacity of fibroblasts, non-patient derived fibroblast cell lines VH7 and BJELR as well as the primary pancreatic CAF were analyzed by conducting a standardized migration assay on monocultures. All three types of fibroblasts maintained a reproducible degree of migration (Figure 2). However, there was a significant difference in the capacity of self-induced cell migration. Multiple comparison analysis revealed a remarkable difference regarding the migration capacity in monoculture between VH7 and CAF ($p=0.018$, Table II) and was confirmed by pairwise comparison ($p=0.009$, Table II).

Effect of PANC-1 on fibroblast migration. In the next set of experiments, we analyzed the migration characteristics of the included types of fibroblasts in indirect coculture with the

Table II. Overview of the analysis results. Listed are the conducted statistical tests with the corresponding *p* values based on data obtained from the measurements presented in this study. PANC-1=P.

Migration fibroblasts (monoculture)	<i>p</i> -Value	Migration PANC-1 (indirect co-culture with fibroblasts)	<i>p</i> -Value
Kruskal-Wallis test	0.007	Kruskal-Wallis test	0.064
Dunn's multiple comparisons test		Dunn's multiple comparisons test	
VH7_ vs. BJeLR_	0.432	P_ vs. P_VH7	0.629
VH7_ vs. CAF_	0.018	P_ vs. P_BJeLR	>0.999
BJeLR_ vs. CAF_	0.998	P_ vs. P_CAF	0.025
Mann Whitney test		Mann Whitney test	
VH7_ vs. CAF_	0.009	P_ vs. P_CAF	0.001
Migration of fibroblasts (indirect co-culture with PANC-1)	<i>p</i> -Value	IL6 concentration in medium supernatant	<i>p</i> -Value
Kruskal-Wallis test	0.211	Kruskal-Wallis test	0.001
Dunn's multiple comparisons test		Dunn's multiple comparisons test	
VH7_ vs. VH7_P	>0.999	CAF Monoculture vs. Indirect Coculture	0.269
VH7_ vs. BJeLR_	>0.999	CAF Monoculture vs. Direct Coculture	0.022
VH7_ vs. BJeLR_P	>0.999	CAF Monoculture vs. PANC-1 Monoculture	0.765
VH7_ vs. CAF_	>0.999	Indirect Coculture vs. Direct Coculture	0.940
VH7_ vs. CAF_P	0.238	Indirect Coculture vs. PANC-1 Monoculture	0.020
VH7_P vs. BJeLR_	>0.999	Direct Coculture vs. PANC-1 Monoculture	0.002
VH7_P vs. BJeLR_P	>0.999		
VH7_P vs. CAF_	>0.999	Mann Whitney test	
VH7_P vs. CAF_P	>0.999	CAF Monoculture vs. Indirect Coculture	0.005
BJeLR_ vs. BJeLR_P	>0.999	CAF Monoculture vs. Direct Coculture	0.009
BJeLR_ vs. CAF_	>0.999	Indirect Coculture vs. Direct Coculture	0.012
BJeLR_ vs. CAF_P	>0.999	CAF Monoculture vs. PANC-1 Monoculture	0.009
BJeLR_P vs. CAF_	>0.999		
BJeLR_P vs. CAF_P	>0.999		
CAF_ vs. CAF_P	>0.999		
Wilcoxon matched-pairs signed rank test			
VH7_ vs. VH7_P	0.250		
BJeLR_ vs. BJeLR_P	0.500		
CAF_ vs. CAF_P	0.098		

Bold values indicate statistical significance.

established PDAC cell line PANC-1 (Figure 3). We were not able to detect any significant effect of PANC-1 cocultures on the migration of studied fibroblasts when compared to migration in monoculture. In pairwise comparison however, we observed a clear trend of increasing CAF migration when cocultured with PANC-1 when compared to the monoculture ($p=0.098$, Table II).

CAFs increase migration of PANC-1 in indirect co-culture. We subsequently analyzed the effect of fibroblasts on the migration of PANC-1. For this, PANC-1 cells were studied either in monoculture or in indirect coculture with VH7, BJeLR and CAFs, respectively (Figure 4). PANC-1 cells showed a consistent and reproducible migration in monoculture (Figure 5). When cultured in indirect coculture

with the two non-patient derived tumor associated cell-lines VH7 and BJeLR, we did not detect any change in cell migration of PANC-1 cells. After coculturing with CAFs however, the migration of PANC-1 cells increased significantly, when tested with multiple comparison ($p=0.025$, Table II) and in pairwise correlation ($p=0.001$, Table II).

Significant increase of IL6 in CAF-PANC-1 co-culture. To further understand the underlying paracrine effects on tumor cell migration, we analyzed the IL6 concentration in the supernatant of PANC-1- and CAF monocultures as well as in direct and indirect co-cultures (Figure 6). Unlike CAFs, PANC-1 cells did not show secretion of IL6. We therefore concluded that in all cell culture setups, IL6 is secreted solely from CAFs. Further, PANC-1 cell presence in the coculture setups led to a significant increase in IL6 secretion by the CAFs both in indirect coculture (multiple comparison: $p=0.064$, pairwise comparison: $p=0.005$, Table II) as well as in direct coculture (multiple comparison: $p=0.0026$, pairwise comparison: $p=0.009$, Table II).

Figure 3. Migration of fibroblast cell lines and primary cancer associated fibroblasts (CAFs) in indirect coculture with PANC-1. The fibroblasts VH7 (n=3), BJELR (n=3) and CAF (n=9) were cultured in mono-culture versus indirect coculture with PANC-1 (P) and after 24 h incubation, the migration of the fibroblasts was analyzed. We could demonstrate a trend of an increased migration when CAFs were cocultured with PANC-1 (CAF_P). Cell-name followed by the character “_” were in the top chamber of the transwell set-up, cell-name preceding the character “_” were in the bottom chamber of the transwell set-up.

Discussion

In this study, we aimed to better understand the impact of CAFs on pancreatic carcinoma cells using PANC-1 carcinoma cell line, as well as primary and established cell culture models of human fibroblasts, that mimic the tumor microenvironment of PDAC (43). There is an ongoing debate about whether CAFs actually possess an innate malignant potential (44). Recent studies by our group have already demonstrated that CAFs can confer chemoresistance when co-cultured *in vitro* (38). In the present study, we compared different types of human fibroblasts: patient derived malignant CAFs, fibroblasts with activated oncogenes and inhibited tumor suppressors (BJELR), and benign fibroblasts. We observed that CAFs exhibit malignant features as they migrate unlike other fibroblasts and also, exclusively promote the migration of PDAC cells. Thus, they evidently play an active role in cell migration and we would therefore refute their former characterization as ‘bystander cells’ (45).

Genetic alterations in the RAS family and p53 have been discovered to be critical in pancreatic cancer development and progression (46). This has been mainly established on the duct/acinar cell fraction. Stromal cells have not yet been analyzed regarding their RAS and p53 characteristics. Lately, it has been theorized that these alterations may play a role in CAF function as well. Several studies have detected a p53

Figure 4. Migration of PANC-1. The migration of PANC-1 (P) after 24 h was observed in monoculture (n=10) and in indirect coculture with different types of fibroblasts (VH7, n=3), BJELR (n=5) and cancer associated fibroblast (CAF) (n=5). Migration of PANC-1 increases significantly when in indirect coculture with primary pancreatic cancer fibroblasts (P_CAF). Cell-name followed by the character “_” were in the top chamber of the transwell set-up, cell-name preceding the character “_” were in the bottom chamber of the transwell set-up.

alteration in CAFs that modify their functioning and enhance the malignant features of their respective cancers (47). This non-autonomous effect is initiated *via* regulation of cancer-promoting genes and secreted proteins (35, 48), also in line with a different study, which revealed a direct link between cancer cells and CAFs in chemotherapy response of lung cancers based on p53 dependence (49).

RAS mutations (particularly *KRAS*) are known to be oncogenic, and in combination with p53 alterations suffice to develop tumors in both, human and genetically engineered mouse models (*e.g.* KPC mice) (50-52). Moreover, HRAS-transformed mouse fibroblasts show increased genomic instability and altered chromosomal protein phosphorylation (53). Murine RAS mutated fibroblasts generated a high local level of proteins involved in tumor growth and angiogenesis (54). Studies suggest that a RAS induced activation of CAF might be one important underlying mechanism causing this (55). We therefore evaluated the effect of HRAS mutated and p53 suppressed fibroblasts (BJELR) in our experiments. Remarkably, we were unable to detect an increased capacity for cell migration by BJELR fibroblasts, nor found evidence they could enhance the migration of PDAC cells in indirect co-cultures.

Several limitations of this study should be noted: First of all, we chose a two-dimensional cell culture setup in our experiments. For this reason, important factors that might influence cell migration, including fiber composition and alignment of the extracellular matrix scaffold, were not considered. However, the setup essentially facilitated high reproducibility of *in vitro* assays, while considering the

Figure 5. Staining of migrated cells. The three different types of fibroblasts VH7 (A), BJelR (B), cancer associated fibroblast (CAF) (C) and PANC-1 cells (D) were stained following 24 h migration through 8 μm pores (Transwell®-based migration assay).

limited number of available patient derived fibroblasts. This was in part due to the fastidious derivation process with a relatively slow doubling time and early senescence of mesenchymal cells in the primary tumor. However, we believe that working with patient derived primary CAFs is essential, in order to investigate and model the biology of the pancreatic tumor microenvironment. This could be supported by our findings that neither benign, nor genetically modified fibroblasts show significant effect on the migration capacity of PDAC cells. In addition, we focused solely on CAFs, while other stromal components like immune cells or endothelial cells were not included in the analysis. Also, the interaction with vascular structures may play an important

role in the early steps of metastasis. For this reason, experiments that include the investigation of other cell components of the pancreatic cancer microenvironment are warranted.

CAFs in PDAC are known to express α -SMA, a microfilament forming protein particularly active in activated myofibroblasts (56). All CAFs analyzed in the present study were verified to express α -SMA (Figure 1). Activated CAFs are known to produce excessive amounts of ECM in the presence of carcinoma cells (57, 58). However, the increase in migration detected in our experiments must be an additional paracrine effect as there was no direct cell-cell contact with cancer cells. In the densely packed desmoplastic

Figure 6. IL6 concentration in the medium supernatant. IL6 concentration was measured in the supernatant of PANC-1 monoculture (n=3), cancer associated fibroblast (CAF) monoculture (n=3), in indirect (n=4) and direct coculture (n=3) of CAFs with PANC-1. PANC-1 did not show any production of IL6. The presence of PANC-1 in the coculture however, induced a significant increase in IL6 production by CAFs.

stroma, systemic hormonal mediators and cytokines are unlikely to reach the tumor cells *via* the blood stream. Thus, a more local crosstalk must play a predominant role in intercellular communication. To this end, we investigated IL-6, a pro-inflammatory cytokine known to promote migration and metastasis (30). We could verify that CAFs are the major source of IL-6 in the PDAC-CAF axis. PDAC cells did not show IL-6 secretion, while enhanced the capacity of CAFs to produce IL-6, most eminent in direct co-cultures.

The crosstalk between stromal cells and tumor cells has recently been discovered as a potential source of novel therapeutic targets. Preclinical studies investigating the depletion of CAFs revealed promising anti-cancerous effects (59). Yet, this targeted approach remains challenging as the CAFs role in the tumor microenvironment is complex and still under investigation. Depletion even leads to an increase in EMT (60). Therefore, the focus must remain on CAF-dependent pro cancerous pathways, such as IL6/STAT3 signaling. The anti-fibrotic tyrosine kinase inhibitor nintedanib was shown to reduce tumor aggressiveness in intrahepatic cholangiocarcinoma *via* suppression of IL6 and other cytokines (61). Moreover, several novel inhibitors of the IL6/STAT3 pathway, as Stattic (6-Nitrobenzo(b)thiophene-1,1-dioxide) and AZD9150, [a signal transducer and activator of transcription 3 (STAT3) antisense] are being investigated in both, preclinical and early phase clinical studies. In combination with other

established chemotherapeutics, a reversed effect of STAT3-mediated chemoresistance was documented (62). Napabucasin (2-acetylfluro-1,4-naphthoquinone or BBI-608), an inhibitor of STAT3 signaling, is currently being investigated in a phase III trial together with nab-paclitaxel and gemcitabine for metastatic pancreatic cancer (63). In addition to chemotherapeutics, targeting the IL6/STAT3 pathway is promoted as a feasible therapeutic approach in the advanced tumor stage. However, to specifically target the invasive properties supported by CAFs, further studies investigating the underlying signaling pathways are needed.

Conclusion

CAFs show the capacity to migrate *in vitro* and also enhance the migration of PDAC cells. This feature is accompanied by an increase in IL-6 production. Non-cancer associated fibroblasts from established cell lines display none of these properties. We therefore conclude that CAFs are a key element of intra-tumoral migration and should, in this context, be investigated as a potential therapeutic target.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

JP, MB, IMS, RBS CCMN and EvH developed the project. EvH performed the experiments. CCMN, EvH, MF and RBS analyzed the results, ARS, EvH, MF, JG and AS contributed to the interpretation of the results. EvH and RBS wrote the paper. MB, MF, IMS, JP ARS, JG and CCMN revised the Paper.

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