ESMO Translational Research Fellowship  
(2011 – 2013)  

Project Title  
‘Molecular characterisation of the stromal microenvironment in pancreatic adenocarcinoma and its role in tumour progression and prognosis’  

Konstantinos Kampsioras  

Final Report  

Host Institute: University of Leeds, Wellcome Trust Brenner Building, St James’s University Hospital, Beckett Street, Leeds, West Yorkshire, LS9 7TF, United Kingdom  
Mentor: Francesco DelGaldo, Head, Scleroderma Research Programme  

Rationale and Aim  

Pancreatic ductal adenocarcinoma (PDA) is one of the most highly fatal cancers, with >95% of those affected dying of their disease. The high mortality is due to the high incidence of metastatic disease at presentation and chemotherapy resistance on the other hand.  

Caveolin-1 (Cav-1) is a major constituent of caveolae and plays a regulatory role in several signalling pathways. Overexpression of Cav-1 has been associated with tumour progression and aggressiveness in pancreatic cancer (Br J Cancer. 2002 Nov 4;87(10):1140-4; J Mol Histol. 2009 Feb;40(1):23-9). Moreover Cav-1 seems to mediate chemo and radio-resistance in different cancer types (Am J Cancer Res. 2011;1(4):521-30 ; Radiother Oncol. 2009 Sep;92(3):362-70). Nevertheless, conflicting results suggesting a different role of the Cav-1 in the development of PDA have been reported recently (Cell Cycle. 2011 Nov 1;10(21):3692-700.)  

The tumour microenvironment plays a previously unrecognized role in human cancer onset, progression and chemo resistance. The host stromal response to an invasive epithelial carcinoma is called a desmoplastic reaction (DR) and is a common feature of PDA. The DR in Pancreatic ductal adenocarcinoma is predominantly characterized by an abundant desmoplastic stroma that is partially induced by activated pancreatic stellate cells (PSCs). In our previous experience we have demonstrated that breast cancer-associated fibroblasts (BCAFs), take on the characteristics of myofibroblasts and promote, through functional down regulation of caveolin-1 and Retinoblastoma
tumour suppressor (RB). Epithelial to Mesenchymal Transformation, a crucial drive to the invasive phenotype [Am J Pathol. 2009 Mar;174(3):746-61]. Additionally, Microarray studies of BCAFs revealed a signature of differentially expressed genes that is strongly associated with the clinical prognosis further suggesting the importance of the role of BCAFs in Breast cancer progression. Here we aimed to determine whether the expression of caveolin could play any role in the progression and chemo resistance of PDA. Our initial aim was to study the role of caveolin-1 in cancer cell in correlation with disease progression and prognosis. Moreover, based on the previous experience on BCAFs we aimed to study the phenotype and putative functional role of PDA associated fibroblasts (PDA-AF) in the progression of PDA lesions.

Specific aims were:
1. to explore the role of caveolin-1 expression in pancreatic cancer progression and chemo resistance
2. to study whether PDA-AF play a role in the progression and drug resistance of PDA cells;
3. to analyse whether any of the genes differentially expressed by PDA-AF can be used as markers of cancer activity, or prognosis or resistance to therapy

**Experimental design**

**IHC staining for caveolin-1:** 15 excision biopsies of pancreatic cancer were stained for caveolin -1 and scored by an independent pathologist (CV).

**Functional experiments in cell lines with different expression level of caveolin 1:** Since our research project was focused on the role of caveolin-1 in the progression and drug resistance of pancreatic adenocarcinoma, we purchased two pancreatic cell lines that according to the literature should have a different level of caveolin-1 expression. The two cell lines are AsPC-1 and BxPC-3. Very importantly AsPC-1 cells are known to derive from malignant ascites of a patient with pancreatic cancer and show high metastatic potential with many visceral deposits in the mice that they were developed. [Tumour Biol 1985; (6)1: 89-98]

Cell biology studies on PDA cells showed that Sonic Hedgehog may be involved in the proliferation and chemo resistance of PDA (Science 2009 Jun 12;324(5933): 1457-61). Moreover, the desmoplastic reaction is known to be mediated by Transforming growth factor beta (TGF-b) which is the major profibrotic growth factor. To determine whether these two molecules could affect PDA cell function We performed functional experiments incubating both cell lines with recombinant Sonic Hedgehog (rSHH) and Transforming Growth Factor-β (TGF-β) at different concentrations to evaluate whether there was a different signalling as far as expression of Gli1, Gli2 and PTCH-1 is concerned, and study the cell migration following a scratch assay.

Since the two pancreatic cell lines express different levels of caveolin-1 we collected their secretome and co-cultured normal dermal fibroblasts with AsPC-1 or BxPC-3 supernatants to evaluate whether the desmoplastic reaction observed in pancreatic cancer could be a direct effect of molecules secreted by pancreatic cancer cells.

**Caveolin silencing** To minimise differences between various cell lines we set out to induce stable knock down of caveolin-1 in BxPC-3 cells that naturally express high levels of caveolin 1. Hence, we were able to measure their proliferating capacity, their migration capacity, chemo sensitivity and their ability to induce a profibrotic microenvironment. We induced stable knock down through
lentiviral infection, which allowed also tracking the cells containing the virus because of constitutive GFP expression (fluorescent in the green channel). The cell line generated from the silencing employing shRNA for caveolin n. 97 was named BX97.

**Functional experiments in cells with silenced caveolin-1:**
- SHH signalling was explored by either the use of recombinant SHH or the transfection with plasmids expressing SHH.
- Migration/Proliferation assay: Scratch assay was used to study the migration-proliferation differences between cells expressing or not caveolin-1. Stimulation of cells with recombinant SHH (rSHH) 2μg/ml was performed and the effect on migration was assessed.
- Proliferation of cells was assessed also by sulforhodamine B (SRB) and Bromodeoxyuridine (BRDU) assays.

**Generation of spheroids:** Subconfluent cell cultures were treated with trypsin and the detached cells were counted, and seeded into specialized 96-well plates (Nano-Culture Plate, Scivax Corporation, Kawasaki, Japan) at a seeding density of 5,000 cells/well, following the manufacturers’ instructions. Briefly, before plating, cells were suspended in specialized medium that was provided with the culture plates. Prior to performing the experiment, several cell densities were tested to select the most appropriate for the formation of spheroids. The drugs added 48 h later upon cells’ addition to the wells so that to allow the generation of spheroids. Cells were further incubated at 37 °C for up to 240 h (5 days) and images were taken using an inverted microscope equipped with a camera.

**Drug Sensitivity:** In vitro cytotoxic activity was determined by use of the SRB assay. Cell viability was assessed at the beginning of each experiment by the trypan blue dye exclusion method, and was always greater than 97%. For the SRB assay, cells were seeded into 96-well plates in 100 μL medium at a density of 7,000 cells per well, depending on the cell line, and subsequently, the plates were incubated under standard conditions for 24 h to enable the cells to resume exponential growth before addition of the compounds. Chemo sensitivity for a variety number of chemotherapeutics was tested in serial dilutions (Gemcitabine, 5-FU, oxaliplatin, cisplatin, Paclitaxel, Docetaxel). Chemo sensitivity in the 3-D culture was tested in spheroids using different concentrations of two chemotherapeutic agents (5FU and Gemcitabine).

**Xenograft growth models:** Male SCID (NOD.CB17 Prkd scid) mice were maintained under restricted flora conditions in a pathogen-free environment in type III-cages. Male mice, 7–9 weeks old, were injected subcutaneously according to the British practice of bilateral trocar implants at the axillary region. Each inoculum contained 10^6 cells exponentially growing at the time of harvesting. The experiment was performed twice, each group consisting of 5 animals.

**Morphology and immunohistochemistry of tumours:** Tumours were excised, fixed in buffered formalin (4%), embedded in paraffin, and 4-μm sections were prepared. Subsequently, sections were stained with hematoxylin-eosin and examined under a microscope to assess the histological phenotype of the tumour, the type and degree of differentiation.

**Isolation of Pancreatic fibroblasts from surgical specimens:** Human Pancreatic fibroblasts could be isolated from surgical samples using collagenase-D.
**Co culture of pancreatic fibroblasts with cancer cells expressing different levels of caveolin:**
The differential expression of collagen-1, smooth muscle actin and caveolin-1 was assessed in pancreatic fibroblasts when they were treated with Supernatant from cancer cells with different level of caveolin-1. Immunofluorescence was also used to visualise the interaction of the cells.

**Silencing of Caveolin in skin fibroblasts:** hTERT immortalized skin fibroblasts were kindly provided from Dr Del Galdo’s group.

**Co injection of pancreatic cancer cells, with different levels of caveolin-1, with immortalised skin fibroblasts**
For the animal experiments a mixture of tumour cells (pancreatic cancer cells (BXPC-3)); 1 million cells) and immortalised human skin fibroblasts (3 hundred thousand cells) in 100 µl of sterile PBS were co-injected into the flanks of athymic NCr nude mice.

Mice were then sacrificed by asphyxia 8 weeks post-injection; tumours were excised to determine their weight and size as described (Migneco G. et al. Cell Cycle 2010; 9:2412-22). According to the principle of 3R, the number of mice to be employed in these experiments has been calculated as following:

Previously published data on breast cancer animal experiments (Migneco G. et al. Cell Cycle 2010; 9:2412-22) have shown a variability of 30% of tumours width and weight. To calculate a difference of 30% in weight and width with a power of the 80% and alpha < 0.05%, the n for each group is = 4. Two groups were used; first one BX97+ fibroblast; and the second BX MOCK + fibroblasts

**Morphology and immunohistochemistry of tumours:** Tumours were excised, fixed in buffered formalin (4%), embedded in paraffin, and 4-µm sections were prepared. Subsequently, sections were stained with Masson’s trichrome and examined under a microscope to mainly assess stromal reaction

**Results, Conclusions and Future Perspectives**

**IHC staining for caveolin-1 (CAV)**
The first interesting finding that we observed was wide intratumoural heterogeneity of Caveolin-1 expression. Well/moderately differentiated pancreatic cancer areas were CAV negative while in poorly differentiated areas PDA cells stained moderately or strongly positive for CAV-1. These findings were in line with the epidemiologic studies identifying the high expression of caveolin as poor prognostic factor. Nevertheless, we noticed that the stroma surrounding the PDA cells showed a consistently opposite pattern of caveolin expression. Indeed, we found that there was high CAV expression in the stroma around well differentiated areas while the opposite is observed in the poorly differentiated areas (Fig 1). This highlights the need to explore the role of caveolin in both the cancer cells and the stroma in order to find out if in both or in which compartment the cav-1 is prognostic or could predict for chemo resistance.
Figure 1 (A-B). Caveolin-1 expression in moderately differentiated pancreatic cancer area (A) with high expression in the stroma (thin arrow) and no expression in the cancer cells (thick arrow) and poorly differentiated are of the same patient with the opposite pattern (B) (Original magnification 40x).

Functional experiments in cell lines with different expression level of caveolin 1
We did demonstrate the different caveolin expression between the 2 cell lines (BxPC3 and AsPC1) (Fig 2)

Figure 2: Western blot. Differential expression of caveolin-1 between BXPC-3 cells and ASPC-1 cells (in triplicates)

The experiments showed that rShh induced a stronger expression of Gli1 and PTCH-1 mRNA in AsPC-1 cells, as expected, whereas BxPC-3 cells showed inconsistent results (Fig 3a and 3b). At protein level, BXPC3 cells showed, nevertheless, a constitutive expression of Gli1 that we could not observe in AsPC-1 cells. In addition Gli1 and 2 were expressed in BxPC-3 cells (protein expression with Western Blot) but not in AsPC-1 cells, after stimulation with SHH and TGF-β (Fig. 3c)

Figure 3: RT-PCR in duplicates for caveolin-1 (CAV) Gli1 and PTCHD after incubation with SHH 2μg/ml in ASPC1 (A) and BxPC3 (B). Protein expression of Gli1/Gli2 after stimulation with SHH and TGF-β 10 ng/ml(C) (SF=serum free; 0.5%=0.5% RPMI)
In the coculture experiments, both smooth muscle actin (SMA) and collagen-1 are decreased in the fibroblasts treated with SN from both cancer cell lines. (Fig 4) suggesting that maybe other factors irrespectively of the caveolin could influence these changes on fibroblasts.

**Figure 4**

RT-PCR for the expression of caveolin-1 (CAV-1), Smooth muscle actin (SMA) and collagen 1 (COL) in fibroblasts untreated (F BX CTRL and F CTRL) or treated with supernatant from BXPC3 (F BX A and B) (A) and AsPC1 (F AS1 and f As 2), (B) respectively.

Altogether these results were inconclusive and perhaps suggested that the cells lines may have had more than just caveolin expression differences to modulate their behaviour in vitro. Therefore, to determine the role of caveolin in PDA cells proliferation and profibrotic activity we set out to silence caveolin expression in BXPC3 cells, to avoid any further bias.

**Lentiviral induced shRNA expression induced effective and stable silencing of caveolin-1 in BXPC3 cells.**

BxPC3 cells were infected with different titres of caveolin and scrambled shRNA lentiviruses. Lentiviruses contained also resistance factor for Puromycin. Cells were selected by adding 2ug/ml of puromycin for one week and further sorted by FACS for GFP expression to increase cell purity. Cells surviving in puromycin medium and sorted for high GFP expression were used for further experiments.

Figure 5 shows that cells infected with shRNA for caveolin expressed very low levels of caveolin when compared with cells infected with the mock shRNA lentivirus. (Fig 5A-C)
Figure 5. Lentivirus infected cells showing green in green channel due to constitutive GFP expression (A). Due to variable GFP expression (arrow), cells were sorted for high GFP expression by FACS (B).

Differential protein expression of caveolin-1 by western blot (C).

**A.**

**B.**

**C.**

**MOCK BX97**

Functional experiments in cells with silenced caveolin-1:
- The silencing of caveolin-1 induced a lower level of expression of SHH targets such as Gli1 and PTCHD. Nevertheless, stimulation with SHH, by transfection of SHH expressing plasmids, induced a similar modulation of expression of both targets in cells expressing or not caveolin-1. *(Fig. 6)*

Figure 6. Silencing of caveolin induced lower expression of Gli1 and PTCHD (A). Transfection with plasmid expressing SHH showed similar pattern of Gli1 and PTCHD expression (B)
- Migration/Proliferation assay: SHH stimulation and caveolin-1 silencing sorted a similar effect on migration by increasing the migration index at 28 hours by 147% and 155% respectively. Nevertheless, SHH stimulation had an opposite effect on cav-1 silenced cells reducing their migration index by 56%. (Fig 7)

**Figure 7:** Migration-proliferation assay. Silencing of caveolin (C) has similar effect as SHH stimulation on mock cells (B). SHH reverses the effect of silencing (D).

- In BRDU assay, BX97 cells incorporate significantly faster BRDU as compared with Mock and BXPC3, indicating higher proliferation of the cells when caveolin is silenced (p=0.01) SRB assay showed BX97 cells tend to proliferate faster but this was not proved to be statistical significant.
**Generation of spheroids**
No difference in the formation of spheroids could be demonstrated between the 3 cell lines. *(Fig 8)*

**Figure 8.** Spheroid formation. No significant difference could be observed between the three cell lines

**Drug Sensitivity**
PDA cells silenced or not for caveolin were treated at concentrations ranging from 1 to 100 uM for anthracyclines (epirubicin and doxorubicin), 5-FU, gemcitabine, oxaliplatin and cisplatin. Paclitaxel was used at concentrations ranging from 0.01 to 10 uM.

Doxorubicin induced a dose dependent suppression of growth rate as measured by SRB in both cell lines, irrespectively of caveolin 1 expression. Similarly, epirubicin treatment with doses ranging from 1 to 100 uM induced a 50% reduction in growth rate at concentration of 1 uM and nearly 100% at 100uM, irrespectively of caveolin 1 expression. Treatment with the rest of chemotherapeutic agents induced similar results in both cell lines. Altogether these results were very convincing on the lack of any functional effect of caveolin expression in chemo sensitivity on BXPC3 cells. *(Fig 9)* Consistently with SRB experiments, the generation of spheroids was similarly suppressed by all chemotherapeutic agents irrespectively of the amount of caveolin expression. (data not shown).

**Figure 9: Chemosensitivity assay** Results are from one exp. run in triplicates using four concentrations for each drug (10-0.01 for taxanes and 100-1 for the rest), 48hrs incubation and the SRB assay. They cannot be considered conclusive definitely for taxanes and gemcitabine as more concentrations at the lower range need to be tested. Negative numbers denote cytotoxicity.
**PACLITAXEL**

- Concentration (µM)
- % Growth Rate

**GEMCITABINE**

- Concentration (µM)
- % Growth Rate

**OXALIPLATIN**

- Concentration (µM)
- % Growth Rate

**CISPLATINUM**

- Concentration (µM)
- % Growth Rate
**Xenograft growth models**

In vivo modelling is possible. No difference could be observed between the different groups. Take rates were: 80% for BxPC3, 62.5% for MOCK and 90% for Bx97. No statistically significant difference was observed by t-test (Fig 10). Nevertheless, when comparing the growth rate of our xenografts with the ones published in the literature we noticed that BXPC3 cells induced tumours at much slower rate. Given the aggressiveness of PDA in human disease, we were led to consider the injection of BXPC3 cells not representative for the human disease, and therefore for the principle of the reduction and refinement, we decided not to pursue chemo sensitivity experiments in vivo.

**Figure 10:** Tumor Growth rate in Vivo. No difference in growth between BX Mock and BX97 could be noticed.

![Tumors' Growth Rate](image)

**Morphology and immunohistochemistry of tumours**

The findings are similar between the three different types, including the morphology, stromal reaction and focal lymphocytic infiltrate. They resembled moderately differentiated adenosquamous carcinoma. They grew in cribriform and solid formations, which were compactly arranged and had pushing type margins. There was focal minimal chronic inflammatory cell infiltration at the tumour periphery. Stroma was not as abundant as in human pancreatic ductal adenocarcinoma, but a desmoplastic stromal response was nonetheless present. (Fig 11)

**Figure 11:** H&E Immunohistochemistry from xenografts (Magnification 10x). No morphologic difference could be observed between the three groups.
The results described above convinced us on the poor if any role of caveolin expression in PDA cells features. Therefore we set out to preform coculture and co-injectione experiments to study the interplay between PDA cells and stroma.

Co-culture of pancreatic fibroblasts with cancer cells expressing different levels of caveolin

PDA cells with reduced caveolin induced fibroblast expression of col1agen and smooth muscle actin at mRNA level (Fig 12). Consistently, by immunofluorescence we observed that fibroblasts around niches of cells expressing low caveolin were expressing high levels of a-SMA. (Fig 13)

Figure 12. Co-culture of pancreatic fibroblasts (PF) with supernatant(SN) from pancreatic cells without silenced (Mock) of silenced (BX97) caveolin. The differential effect on collagen-1 (COL), smooth muscle actin (SMA) and caveolin (CAV) is depicted.

Figure 13: Coculture of pancreatic fibroblasts (PF) with cancer cells. Cancer cells with decreased level of caveolin seem to induce fibroblast to for a protective “niche” around them (arrow). Red: smooth muscle actin of fibroblast; Blue: DAPI staining for nuclei
Co injection of pancreatic cancer cells, with different levels of caveolin-1, with immortalised skin fibroblasts:
We could obtain tumours in the group we coinjected BX97 cells with immortalised fibroblast. Indeed, 3 of the 4 animals gave tumours (of different size). In the other groups (BX MOCK+ fibroblasts) no tumour growth could be observed. Masson trichrome staining was used to visualise the tumours and the stroma reaction. Moderate desmoplastic reaction could be observed while tumours invaded muscle suggesting an aggressive behaviour. (Fig 14)

Figure 14. Masson’s trichrome staining for tumors derived from the co-injection of BX97 and Immortalized fibroblasts. (A) Moderately/poorly differentiated tumor invading the muscle (arrow). (Magnification 20x). (B) Increased desmoplastic reaction (blue color) in the tumour (Magnification 10x)

Conclusions
Although expression of caveolin has been considered a prognostic marker in PDA our extensive in vitro and in vivo studies have shown that caveolin expression in PDA cells has little or no substantial effect on tumour growth and sensitivity to chemotherapy. These results, in view of the well observed dichotomy of caveolin expression between tumour and stroma, rather suggest that if there is any effect of caveolin in PDA prognosis that is potentially to be attributed to the mirror level of expression in pancreatic stroma. If this was the case, PDA would no be so different from other tumour models, such as breast cancer, in which the role of caveolin expression in the stroma has been well established. Notably, in PDA the amount of desmoplastic reaction is a lot more pronounced than in the above mentioned breast cancer stroma In this sense, considered the inverse correlation of caveolin expression and survival in breast cancer, the abundance of the desmoplastic reaction and the poor expression of caveolin in the stroma of PDA may be one of the causes of the dismal prognosis of PDA.

Future Perspectives
To further explore this hypothesis we are setting out to study co-injection models in which the fibroblasts injected are silenced or not for caveolin. Although the experiments planned will extend beyond the end of my fellowship, it is worth to mention that this ESMO fellowship, besides giving me the possibility to train in cell biology, molecular biology and animal biology fields, which I could have not pursued without support, it did catalyse collaboration at international level of 3 different scientific groups. We are currently using the results of this fellowship as preliminary data for grant application to obtain funding for the stroma studies.
Co culture experiments of skin fibroblasts with different levels of caveolins-1 with pancreatic cancer cells are in progress to assess differential tumour growth and chemo sensitivity in vitro and in vivo.

Collaboration with Prof Melcher Laboratory (University of Leeds) has been established to study the role of caveolin in the ability of oncolytic viruses to infect pancreatic cell lines with different levels of caveolin-1 expression.

**List of Publications Resulting from the Grant**

EACR-22, (Barcelona, Spain 7 -10 July 2012)

**Selection of Courses & Workshops and Scientific activities performed During the Fellowship**

- European Multidisciplinary Cancer Congress, Stockholm (23 – 27 Sept 2011)
- In March 2012 Dr DelGaldo was invited to University Hospital of Larissa and presented “Pancreatic Cancer Fibrosis. More than a stromal reaction”. This was the launch of the second year of the Fellowship and the establishment of collaboration between my home Institute and University of Leeds
- “Methods in Clinical Cancer Research” (23-29 Jun2012, Flims, Switzerland). A phase I trial was formulated and concluded during the Course, concerning the combination of a novel SHH inhibitor with radiotherapy in pancreatic adenocarcinoma
- ESMO 2012 Congress (8 Sep - 02 Oct 2012, Vienna, Austria)
- XIV National AIOM Congress (26-29 Oct 2012 Rome, Italy)- In the Educational Session of Young Oncologists of AIOM I presented: “Translational Research Fellowship - sharing the experience"
- ESMO Preceptorship on Gastric cancer” (Berlin-Germany 7-8 Dec 2012)
- We were awarded a Research Grant from the Hellenic Society of Medical Oncology (HeSMO) This grant gave support for consumables of the experiments of chemo sensitivity in vitro and animal studies in vivo.

**Acknowledgments**

Alan Anthoney *(Dept of Oncology, The Leeds Teaching Hospitals NHS Trust, Leeds, UK)*

Gemma Migneco *(Leeds Institute of Molecular Medicine; University of Leeds, Leeds, UK)*

Caroline Verbeke *(Dept of Laboratory Medicine, Karolinska Institute Stockholm, Sweden)*

Alison Cairnes *(Dept of Histopathology The Leeds Teaching Hospitals NHS Trust, Leeds, UK)*

Andy Smith *(Dept of Surgery The Leeds Teaching Hospitals NHS Trust, Leeds, UK)*

Natalia Riobo *(Dept of Biochemistry, Thomas Jefferon University, Philadelphia, PA-USA)*

Christos N. Papandreou *(Department of Medical Oncology; University Hospital of Larissa; Greece)*

Konstantinos Dimas *(Dept of Pharmacology, Faculty of Medicine; Thessaly University; Larissa; Greece)*

Eleana Hatzidaki *(Dept of Medical Oncology; University Hospital of Larissa; Greece)*