Desmoplastic stromal cells modulate tumour cell behaviour in pancreatic cancer
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**Figure 3.3**

**Figure 3.3**: H&E stain of organotypic sections (full length) consisting of PS1 alone (a) and DEC/PS1 ratios (b). There were no observable changes in cellularity or invasion.
Figure 3.4

Figure 3.4: H&E sections of organotypics consisting of Capan1/PS1 cells in specific ratios. An increase in cancer cell layer and an increase in invasion into the matrix was observed.

Figure 3.5

Figure 3.5: H&E stain of organotypic sections consisting of AsPc1 and PS1 cells in specific ratios. Similar observations as seen in Capan1 / PS1 organotypics as above.
Figure 3.6: Box and whisker plots of data for gel length (a-d). Summary statistics comprising of fractional polynomial regression lines are depicted in (e) showing a decrease in the length of the gel as the proportion of stellate cells increased. The maximum effect was seen when the stellate cell proportion was in the range of 0.6-0.8.
Figure 3.7: Box and whisker plots of data for gel thickness (A_D). Summary statistics comprising of fractional polynomial lines are depicted in (E) showing an increase in the thickness of the organotypic gel as the proportion of stellate cells increase. Arrows indicate the range where the changes were most significant.
Figure 3.8: Box and whisker plots of data for fold change of cancer cells (A-D). Summary statistics comprised of fractional polynomial lines are depicted in (E). A marked increase in fold change was seen to commence when the starting proportion of stellate cells was in the range of 0.6 to 0.8.
3.3 Effects of stromal cells on cancer cell proliferation, apoptosis and invasion

The increase in absolute cell number prompted further investigation to study effect of stellate cells on cancer cells with respect to proliferation and apoptosis. It was observed that increasing stellate cell proportion had a pro-proliferative and an anti-apoptotic effect on cancer cells as determined by the percentage of Ki67 and cleaved caspase-3 (Figures 3.9 & 3.10) positive cells in each ratio. This effect was most pronounced when the proportion of stellate cells was in the range of 0.66 to 0.83 (Figures 3.10 (Ki67) and 3.12 (Caspase)). In oranotypics consisting of normal ductal (DEChTERT) epithelial cell and stellate cell mixture, this effect was not evident and there was no measurable change in the fraction of proliferating or apoptotic epithelial cells (Figures 3.10 and 3.12). Similarly, in gels consisting of cancer cells alone, such pro-survival effect of stellate cells was absent. This clearly demonstrated the specific cancer-stellate cell interaction leading to enhanced cancer cell growth and proliferation.

In addition to the marked pro-survival and anti-apoptotic effect of stromal cells on the cancer cell, it was also observed that stellate cells increased single cell invasion as well as cohort invasion (2, 208) of cancer cells into the underlying matrix (Figure 3.13). This was determined by counting the absolute number of single cancer cells and cohorts of cells invading the gel matrix. Distinction between cancer cell and stellate cell in the matrix was made by staining for epithelial marker cytokeratin(Figure 3.13). As the proportion of stellate cells increased, an increase in both single cell and cohorts of cancer cells was observed and once again this effect, upon quantification, was maximal at stellate proportion of 0.66-0.83% (Figure 3.14). However, no invasion was observed in gels with normal epithelial – stellate cell mixture or in cancer cell alone gels, reiterating the exclusive effect of stellate cells upon cancer cell invasion, in addition to, as described above, its pro-survival effect.
Figure 3.9. **Stellate cells have pro-survival effect on cancer cells.**

Immuno-fluorescence staining was performed to look at the proliferation specific marker Ki67 (green) across all ratios and percentage of non-invaded cancer cells (DAPI: blue) positive for the marker was calculated in 6 random high power fields per gel (at least nine organotypics gels were constructed for each ratio, n=54). Capan/PS1 ratios (a) and AsPc1/PS1 ratios (b).
Figure 3.10: Quantification of proliferation. Graphs a-d represents the distribution of the data in box-and-whisker plots (median and inter-quartile range (box) as well as full range (whisker + outliers)) for various gels, experiments and controls.

The fractional polynomial regression model (e) demonstrate that increasing the proportion of stellate cells caused an increase in the percentage of proliferating cancer cells when cancer cells and stellate cells are co-cultured with the maximal effect observed at a starting stellate cell proportion of 0.66-0.83 (arrows). Furthermore, epithelial-stellate or cancer cell alone gels demonstrated no change in the proliferative fraction.
Figure 3.11: Stellate cells have an anti-apoptotic effect on cancer cells.

Immuno-histochemical staining was performed to look at the apoptotic specific marker cleaved caspase-3 across all ratios and percentage of non-invaded cancer cells positive for the marker was calculated in 6 random high power fields per gel (at least nine organotypics gels were constructed for each ratio, n=54).
Figure 3.12: Quantification of apoptosis. Graphs a-d represents the distribution of the data in box-and-whisker plots (median and inter-quartile range (box) as well as full range (whisker + outliers)) for various gels (experiments and controls).

The fractional polynomial regression model (e) demonstrate that increasing the proportion of stellate cells caused a decrease in the percentage of apoptotic cancer cells when cancer cells and stellate cells are co-cultured with the maximal effect observed at a starting stellate cell proportion of 0.66-0.83 (arrows). Furthermore, epithelial-stellate or cancer cell alone gels demonstrated no change in the apoptotic fraction.
Figure 3.13: Stellate cells have pro-invasive effect on cancer cells

In order to analyse invasion, the gels were stained for epithelial cell specific marker cytokeratin (Capan 1/PS1 shown in A) to distinguish cancer cells from stellate cells and number of both single cells and cohorts (cell clumps of >2) invading into the ECM gel was calculated. An increase in both cellular and cohort invasion was evident when there was an increase in stellate cell proportion.
**Figure 3.14: Stellate cells have pro-invasive effect on cancer cells**

On quantifying invasion, an increase in both cellular and cohort invasion was evident when there was an increase in stellate cell proportion with maximum invasion seen when stellate cell proportion was 0.66-0.83. Graphs a-d show box and whisker plots. Graphs e and f represent fractional polynomial regression lines with shaded areas showing 95% CI.
3.4 Effect of stromal cells on expression of well-validated molecular (E-cadherin, β-catenin and ezrin) changes within cancer cells

Based on our (190, 201) and other laboratories'(213, 214) experience on the involvement of key survival (β-catenin(190, 201, 215, 216)) and cell-cell adhesion (E-cadherin(190, 214, 216-219)) molecules, which are interlinked during wnt signalling(214), epithelial-mesenchymal transition(213, 220, 221) (EMT) as well as pro-migratory(3, 214, 216, 218, 219, 222) phenotype of cancer cells, these two molecules were further explored specifically in the context of cancer cell-stellate cell organotypic cultures. In order to avoid the confounding effect of heterogeneous cellular migration (single cell and collective), as well as possible EMT, the non-invaded sheet of epithelial cells above the epithelial cell-stromal cell-gel interface was assessed. Immunofluorescence staining for the proteins was employed across all the ratios and the expression of these proteins was analysed by measuring the intensity of green fluorescence per non-invaded cancer cell using Image J software. The resulting data demonstrated a biphasic expression of E-cadherin and β-catenin per cancer cell prior to invasion (Figure 3.15). As the proportion of stellate cells increased, initially expression of both molecules (E-cadherin (Figure 3.16 a-d) and β-catenin (Figure 3.17 a-d)) increased, followed by a significant decrease when the critical stromal cell proportion was 0.66-0.83 was reached. Finally as the stromal cells increase further (>0.9) there is return of the expression of these molecules to the basal level (i.e., when cancer cells are cultured alone). Importantly, the maximal reduction of expression of these molecules was demonstrable when the cancer cells proportion was less than stellate cells. This change was notably absent in control gels consisting of cancer cells alone. These data offer explanations to many of the conflicting observations made by various laboratories worldwide about the increase as well as decrease of E-cadherin and β-catenin expression in distinct cancers, both in vivo(190, 201, 223) and in vitro(213, 214, 224).

Ezrin is a plasma membrane-cytoskeletal linker protein and has been shown to affect cell shape, polarity, adherence and migration (225). It has been shown previously in our lab that ezrin
plays an important role in modulating cancer cells into a more invasive phenotype (226) and the presence of stellate cells altered its expression as observed previously (190). Immunofluorescence staining for ezrin was carried out across all ratios and the intensity per non-invaded cancer cell was calculated as described above. A decreasing trend of ezrin expression was observed as the proportion of stellate cells increased; however, this was not statistically significant (Figure 3.18).

**Figure 3.15**

![Image of Figure 3.15 showing expression of E-cadherin, β-catenin and Ezrin in cancer cells on exposure to stellate cells.](image)

**Figure 3.15. Expression of E-cadherin, β-catenin and Ezrin in cancer cells on exposure to stellate cells**

To further study the possible mechanisms involved in pro-survival and pro-invasive capabilities of cancer cells, organotypic gels were stained by immune-fluorescence for cell-cell adhesion proteins E-cadherin, Ezrin and β-catenin. The intensity of green fluorescence of the non-invaded cancer cells (above the white line) was determined in Image J software. Presence of stellate cells decreased the cancer cell expression of E-cadherin and β-Catenin. The expression of ezrin showed a trend in decrease but was not statistically significant.
Figure 3.16. Expression of E-cadherin in cancer cells when co-cultured with stellate cells.

The expression of E-cadherin was determined by calculating the green fluorescent intensity per non-invaded cancer cell by using ImageJ software. Graphs a-c represent data as box and whisker plot in experiments (Capan1/PS1 and AsPc1/PS1) and controls (Capan1 only). Graph d represents fractional polynomial regression lines for the experiments and controls indicating a biphatic expression with a significant decrease in E-cadherin expression when the stellate cell proportion ranges between 0.6-0.8.
Figure 3.17

Figure 3.17. Expression of \( \beta \)-Catenin in cancer cells when co-cultured with stellate cells.

The expression of \( \beta \)-Catenein was determined by calculating the green fluorescent intensity per non-invaded cancer cell by using ImageJ software. Graphs a-c represent data as box and whisker plot in experiments (Capan1/PS1 and AsPc1/PS1) and controls (Capan1 only). Graph d represents fractional polynomial regression lines for the experiments and controls indicating a biphasic expression with a significant decrease in \( \beta \)-Catenin expression when the stellate cell proportion ranges between 0.6-0.8.
Figure 3.18. Expression of Ezrin in cancer cells when co-cultured with stellate cells.

The expression of Ezrin was determined by calculating the green fluorescent intensity per non-invaded cancer cell by using ImageJ software. Graphs a-c represents data as box and whisker plot in experiments (Capan1/PS1 and AsPc1/PS1) and controls (Capan1 only). Graph d represents fractional polynomial regression lines for the experiments and controls. Although the expression of ezrin showed a decreasing trend, the results were not statistically significant.
3.5 Changes in cancer cell gene expression profile is mediated by stellate cells

In order to explore the molecular mechanisms of the pro-survival and pro-invasive phenotype Gene-expression micro-array analysis was performed to identify differential expression of genes in cancer cells (Capan1) exposed to stellate cells versus cancer cells alone. As described, cancer cells were cultured on organotypic gels embedded with or without stellate cells and laser micro-dissection were used to capture the cancer cells (Figure 2.2). RNA extracted from the laser captured cancer cells was used for microarray analysis (Figure 3.19 and Table 3.1).

The gene-expression microarray analysis confirmed the transcriptomic change, targeting multiple intracellular pathways, within cancer cells upon exposure to stellate cells (Gene Expression Omnibus website (accession number GSE 36776)). In the pancreatic cancer model, 146 probes demonstrated differential expression which constituted 126 genes (Appendix Table 2), Ingenuity Pathway Analysis, highlighted statistically highly significant changes in gene-expression mediating key cellular functions such as cell-cycle and proliferation, cell movement and death, cell-cell signalling, and inflammatory response mediated through multiple signalling cascades for pancreatic cancer cells. Similar data was obtained as a result of collaborative work with Eric Sahai and Claudia Andl’s laboratory and comparative study revealed interesting inferences (Figure 3.20 and 3.21), which have been detailed in the discussion section. Briefly, there were a number of common pathways that were significantly affected between the three models (Figure 3.22 a) but none or only a few genes were found to be commonly involved between them (Figure 3.22 b and 3.23).
**Figure 3.19:** RNA quality was determined by running the extracted sample in the agilent bio analyser. An example of one of the samples indicating 18s and 28s fragments along with electrophoresis blot is shown here.

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</tbody>
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**Table 3.1:** Table showing the RNA quantity and quality (260/280 ratio) including the RNA integrity number (RIN) of all the samples used for hybridising onto the microarray chip.
Gene expression analysis was performed to identify differentially expressed genes in cancer cells exposed to stellate cells in comparison to cancer cells alone (pancreatic and skin squamous cell cancer).

Heatmaps of differentially expressed genes from cancer cells exposed to stromal cells are shown in (a) for pancreatic cancer organotypic and in (b) for skin squamous cell cancer organotypic cultures.
Figure 3.21: For oesophageal cancer organotypics (a), comparisons were made between hTERT-immortalised normal epithelial cells expressing wild-type E-Cadherin (Ecad) (wild-type) or dominant negative E-cadherin (EC) (suggesting loss of E-cadherin) and dominant negative Transforming Growth factor β Receptor II (ECdnT) (TGFβRII) all of whom were exposed to stromal cells (embryonic oesophageal fibroblasts) as described. The Venn diagram (b) the similarities between the differentially expressed genes in the three cell types.