"Bone-stromal cells up-regulate tumourigenic markers in a tumour-stromal 3D model of prostate cancer"

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Introduction

Prostate Cancer (PCa) is one of the most commonly diagnosed cancers, and has a high propensity to metastasise, especially to the bone. Current *in vitro* techniques primarily focus on the cancer cells in isolation, therefore we attempted to develop a 3D co-culture model between PCa (PC3) and Bone Stromal (HS5) cells.

The α6 and β1 integrin subunits have been implicated in the metastasis to bone [1], as well as in the regulation of cell Epithelial-Mesenchymal Plasticity (EMP) [2]. This lead us to use inhibition assays in this model to study their role in EMP, specifically using PCa-Bone interactions. Underr inhibition conditions we investigated the morphology and invasion rates, as well as expression of E-Cadherin, N-Cadherin, and CXCR7 in mono- and co-cultures.

Our results indicate that co-culture models could become an important tool in understanding how the cancer cells interact with niche micro-environments, and how this may influence the effectiveness of therapeutics.

Methods

PC3 (PCa cells isolated from a metastatic bone tumour) and HS5 (Bone stromal) cells were seeded on top of BD Biosciences Matrigel™ and maintained for up to 9 days in RPMI +10% FBS.

PC3 and HS5 cells were plated either in isolation (mono-culture) or together (co-culture).

Using a variety of techniques, including light and immunofluorescent microscopy, western blot and densitometric analysis, and transwell invasion assays, cells were analysised for morphology, invasive capabilities, and alterations in protein expression.

The α6 and β1 integrin subunits were inhibited using well tested inhibitory monoclonal antibodies, GoH3 (α6, sc-19622) and P5B2 (β1, sc-13590) at a concentration of 2 µg/mL. These antibodies were incorporated into the Matrigel during polymerisation, and were also included in media during media changes.

For immunofluorescence, both primary and secondary antibodies were used at 5 µg/mL.

Challenged media assays involved the application of growth media to either PC3 or 3T3 (fibroblast "control") cells for 24 hours before removing the media and applying it to HS5 cells.

Results

1. Cell morphology of PC3 and HS5 cell lines in singular and co-culture

In order to characterise the cellular phenotype of PC3 and HS5 cells in 3D mono- and co-culture we utilised light microscopy and immunofluorescence techniques.

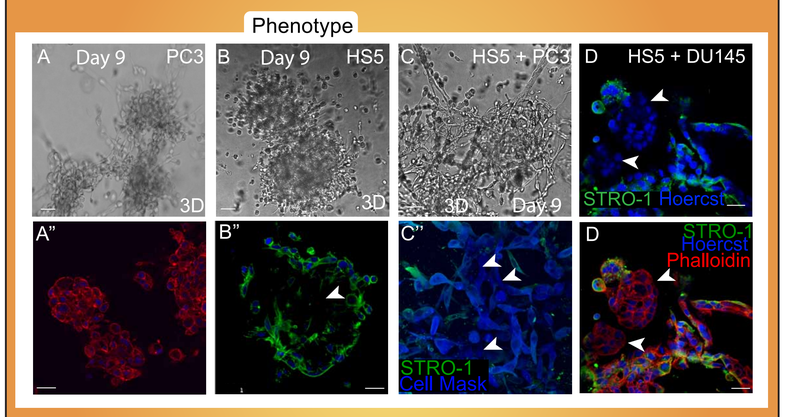

Figure 1. Using light microscopy, phenotypes of both PC3 (A) and HS5 (B) are similar; both form loose aggregates with processes radiating from them. Further investigation using immunofluorescence revealed that that PC3 (A') grew in more solid "grape-like" masses, while HS5 (B') were smaller and grew in loose "meshwork" aggregates. In co-culture (C,C') both cells types interact with each other, disrupting the formation of the typical structures found individually, and making them more disorganised. This indicates that there is an interaction between these two cell types, however does not reveal the full extent, therefore we went on further to evaluate the expression of key EMP proteins. Scale Bar = 40µm.

2. Inhibition of α6 and β1 integrins influences phenotype

Integrins are cell-cell and cell-matrix adhesion molecules, important in conveying signals in to and out of the cell [1]. Therefore we sought to determine if the α6 and β1 integrins were required for PC3-HS5 communication and enabling the changes in phenotype.

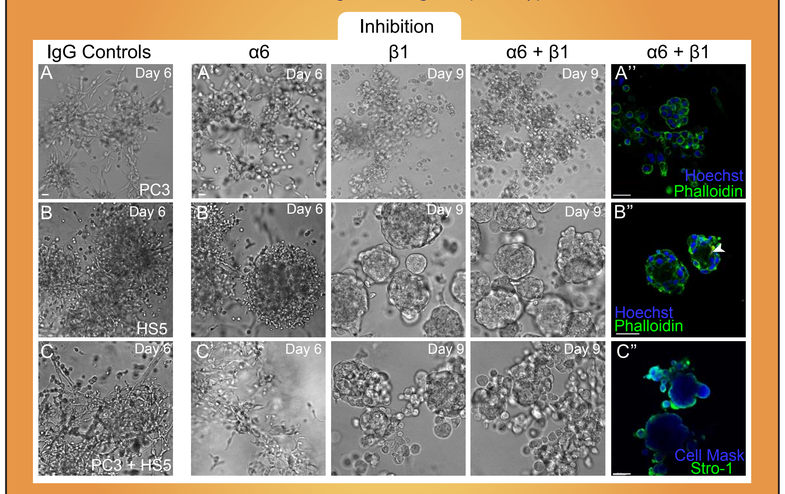

Figure 2: The phenotypic effect of α6 and β1 inhibition was investigated using DIC and immunofluorescence. Effects were more pronounced under β1 inhibition than under α6 inhibition (A', B', C'). PC3 cells lose their stellate morphology and assume a grape-like phenotype (A'), while HS5 assume a more epithelial-like phenotype (B'), with polaristation and the formation of acini (B'', arrow). In co-culture cell-cell contacts were reduced in α6 inhibition, while under β1 and α6+β1 inhibition cells formed tight compact masses with no acini formation (C', C''). Scale Bar = 40 µm.

3. Inhibition of α6 and β1 integrins influences invasion

We next sought to investigate whether cell invasion and proliferation was influenced under co-culture and integrin inhibition conditions, using a transwell invasion and Alamar Blue assays respectively. Laminin was also included as it is a major consitient of the bone micro-environment

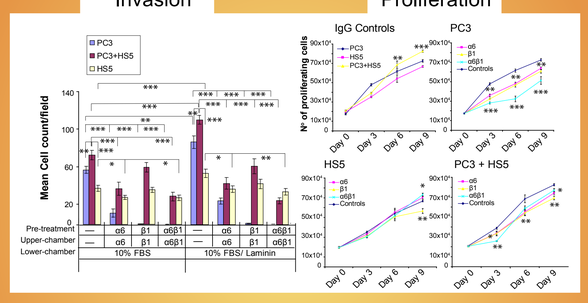

Figure 3: Inhibition of α6 and β1 integrins led to changes in invasive potential and proliferation. The invasive potential and proliferation behaviour of each cell culture was investigated under integrin inhibition. Co-cultures consistently invaded and proliferated at a higher rates than either PC3 or HS5 in control conditions. Invasion by PC3 cells was completely abolished, while co-culture appeared to protect the PC3 cells from the effects of integrin inhibition. Similarly, under β1 or α6β1 inhibition, proliferation rates were significantly reduced in monocultures of PC3 cells. Addition of HS5 cells (co-culture) resulted in attenuated integrin inhibition effects.

4. Alterations of E-Cadherin in both Co-culture and integrin inhibition conditions

E-Cadherin is a marker of Epithelial cell type and it's loss is considered a classic biomarker for an epithelial to mesenchymal transition, which is essential for tumour metastasis [3].

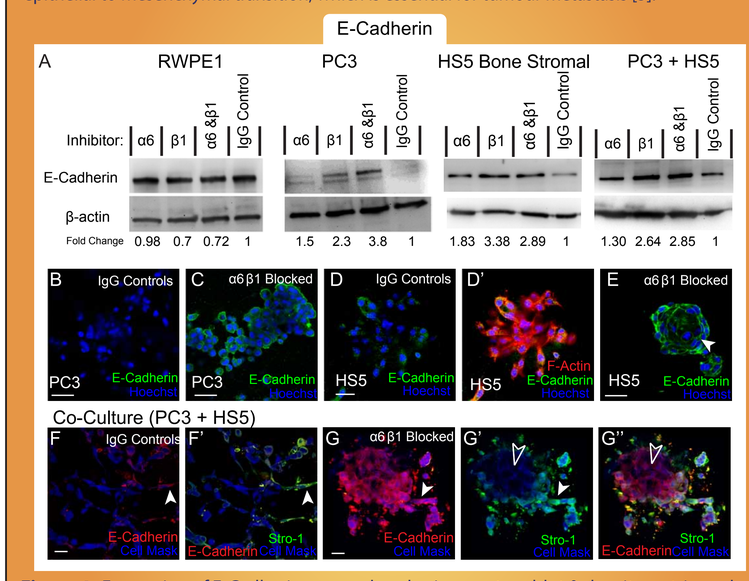

Figure 4: Expression of E-Cadherin was analysed using western blot & densitometric analysis (A), and immunofluorecent techniques (B-G''). RWPE1 cells were used as a control to demonstrate normal E-Cadherin expression in the prostate, and was only minimally effected by integrin inhibition (A). PC3 and HS5 cells were seen to up-regulate expression in α6, β1 and α6+β1 inhibition (A; 1.5-3.8 fold greater), which was localised to the cell membrane (C, E). HS5 highly organised acini-like structures under α6+β1 conditions (E; arrow). In co-cultures, expression was primarily in HS5 cells under control conditions (F, F'; arrow), but was expressed by both PC3 and HS5 when α6+β1 was inhibited (G-G''). Scale bar =40 µm

5. Alterations of N-Cadherin in both Co-culture and integrin inhibition conditions

N-Cadherin is associated with neuronal cell type and is also expressed by many mesenchymal cells. An increase in expression has more recently become associated with a transition from epithelial to mesenchymal [4].

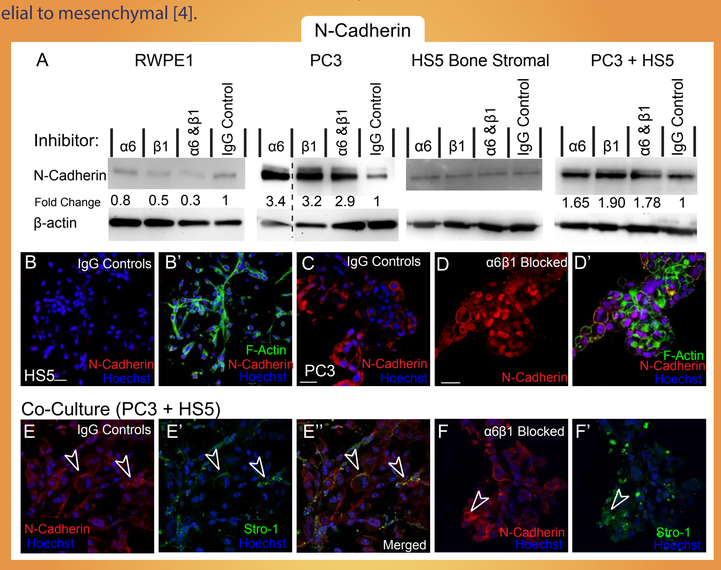

Figure 5: Expression of N-Cadherin was analysed using western blot & densitometric analysis (A), and immunofluorecent techniques (B-E''). RWPE1 cells demonstrated minimal N-Cadherin expression, and was down-regulated under integrin inhibition (A). In contrast PC3 cells clearly expressed N-Cadherin on the cell membrane (C). Under Inhibition N-Cadherin with localised primarily to the cell nucleus (D), indicative of a non-functional protein. HS5 cells displayed minimal expression (A-B'), however when co-cultured expression was seen in both PC3 and HS5 (E-E''), indicating that PC3 cells are potentially able to initiate changes in the surrounding bone matrix. Alpha6+β1 inhibition also resulted in an up-regulation in co-cultures (A) seen in both cell lines, however, distribution was seen at the cell membrane (F-F'), indicative of a functional receptor. Scale Bar = 40 µm.

6. Alterations of CXCR7 in both Co-culture and integrin inhibition conditions

CXCR7 is a chemokine receptor that is minimally expressed by the normal prostate, but is expressed in PCa. It recognises SDF-1α and I-TAC, and it may be linked to homing of PCa to the bone, similar to CXCR4 [5].

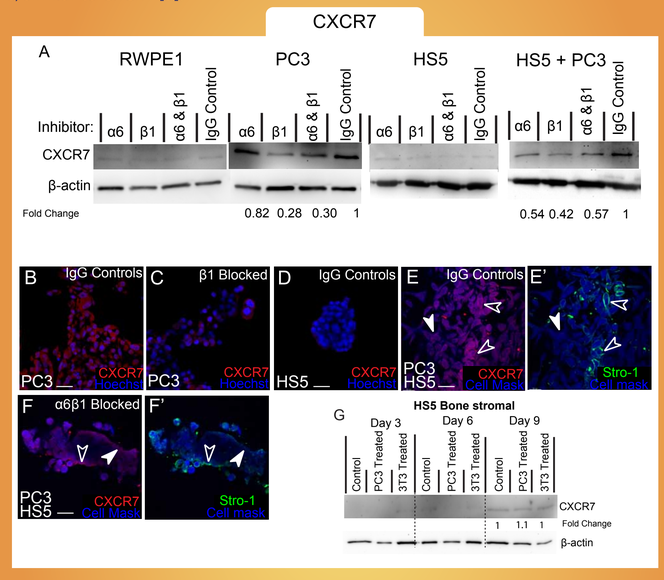

Figure 6: Expression of CXCR7 was analysed using western blot and densitometric analysis (A, G), immunofluorescent techniques (B-F'). Initial western blots reveal that RWPE1 and HS5 express minimal CXCR7, while CXCR7 expression is high in PC3 and in co-culture conditions. These results were confirmed using immunofluorescence, and it was discovered that in co-culture, CXCR7 was expressed by both PC3 (E-E', solid arrow) and HS5 (E-E', hollow arrows). Integrin inhibition down-regulated expression in both isolated PC3 cell cultures and co-cultures, however this effect was less pronounced in co-cultures. This could be due to re-expression by HS5 cells, or could indicate that co-culture diminishes the effect of integrin inhibition. The challenged media assay indicates that excreted factors are not responsible for the up-regulation of CXCR7 in HS5 cells. These results suggest that while the bone stroma does not normally express CXCR7, the presence of PCa cells can induce this expression. Scale Bar = 40 µm.

7. Alterations of N-Cadherin is mediated by secreted factors

We observed that N-Cadherin was expressed by HS5 cells in co-culture, but not in mono-culture, so we sought to ascertain whether this was due to secreted factors from PC3 cells

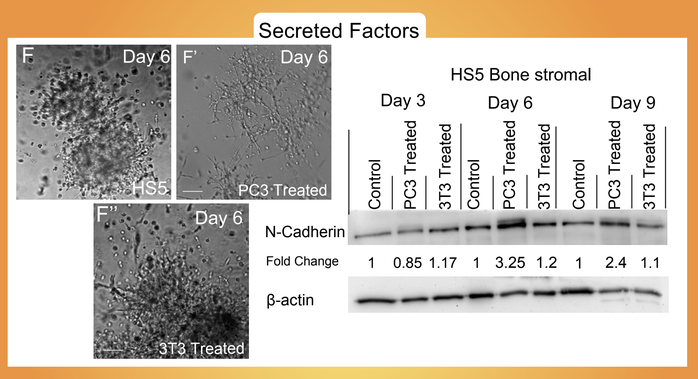

Figure 7: A challenged media assay was used to determine whether up-regulation of N-Cadherin was due to excreted factors from PC3 cells. To investigate this we treated HS5 cells with PC3 treated media over a 9 day time-course. In comparison to untreated HS5 cells (F), HS5 cells grown in PC3 treated media lost their organised phenotype by day 6 in culture and formed irregular shaped clusters with stellate radiating tubular processes, consistent with a metastatic cell-line (F'). These results were PC3 specific as HS5 cells grown in embryonic fibroblastic (3T3) treated media (F'') were unaffected. Moreover, western results confirmed an up-regulation of N-Cadherin expression in HS5 cells when treated with PC3 treated media. Scale Bar = 40 µm.

Discussion

We have developed a cell co-culture model which encapsulates the bone microenvironment in both extracellular matrix (ECM) composition and structure, with the added benefit of the presence of bone stromal support cells. Here we have shown that PC3 and HS5 cells form distinct morphologies when grown in mono-culture, and that this morphology was altered in co-culture. We have also characterised invasion rates, and the expression of key EMT and malignancy-related protiens in this co-culture model.

Furthermore, our data suggests that α6 and β1 integrin subunits mediate behavioural, morphological and protein expression associated with metastatic dissemination in both isolated and co-cultures.

We have also developed an assay to determine whether secreted factors are responsible for protein expression changes, and our results indicate that N-Cadherin expression in HS5 cells is primarily due to secreted factors from PC3 cells, whilst other proteins may be mediated by direct contact factors, such as CXCR7.

Utilising the co-culture model we have shown that the addition of bone derived stromal cells to metastatic cells enhances tumour invasion, and induces protein expression towards a more mesenchymal phenotype. In addition we have shown that PC3 cells may be able to modulate the pre-metastatic niche associated with the bone, as emulated by the HS5 cells, which may in turn provide some benefit to the cancer cells.

These results show that not only is it beneficial to study PCa using a relevant 3D ECM mimetic, but that by using a relevant co-culture system to encapsulate potential cell-cell interactions that occur in *in vivo* situations, more informative data can be obtained.

Acknowledgements

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