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Co-cultures of Mesenchymal Stem Cells and Endothelial Cells as Organotypic Models of Prostate Cancer Metastasis

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KEYWORDS

Prostate cancer, in vitro model, metastasis, bone, microenvironment, organotypic

ABSTRACT

In spite of recognised limitations in capturing species-specific responses and high costs, rodent models remain commonly used in prostate cancer metastasis research, due largely to the lack of available alternatives. We aim to develop an in vitro culture system to study prostate cancer response to a simulated bone microenvironment, which may be used to understand early events in prostate metastasis to bone or for drug screening applications. To achieve this, mesenchymal stem cells and endothelial cells were isolated and co-cultured to form a vascularised bone analogue. Endothelial cells were found to exert osteopotentiating effects on mesenchymal stem cell differentiation, and reciprocal effects by the stromal cells were found to stimulate vasculogenic responses, suggesting the possible utility of this system to elicit three-way interactions between endothelial, mesenchymal and prostate cancer cells. We further developed the use of fluorescently labeled cells which could be used to concurrently track cellular migration, proliferation, as well as morphometric analysis. We demonstrate the concurrent, real-time visualisation of prostate cancer and endothelial cells, which may be useful for evaluation of

spatiotemporal changes at a single-cell level. When prostate cancer cell proliferation on various substrates was measured, it was found that the use of co-culture systems may provide a better reflection of conditions in vivo, highlighting the potential utility as a model system.

1. Introduction

A major difficulty in treatment leading to complete remission of cancer typically arises due to metastatic formation of secondary tumours¹. In what is often termed a "fatal attraction", prostate cancers demonstrate almost exclusive tropism for bone, which is also commonly observed in cancers of the breast, lung, colon, stomach, bladder, uterus, rectum, thyroid and kidney². Much interest has been generated in targeting the metastatic niche in the bone, and clinical trials based on such strategies have yielded sharply contrasting results: the US FDA has approved use of bisphosphonates³, as well as anti-RANKL agents⁴, in the management of breast cancer metastasis, while Phase III clinical trials studying the use of atrasentan for the management of prostate cancer metastasis failed to meet its primary endpoints, despite highly promising pre-clinical studies⁵.

The discrepancy between clinical and pre-clinical findings may partly be explained by the lack of adequate disease models. In murine models of experimental metastasis where prostate cancer cells are intravenously injected, metastatic formation in the skeleton is exceedingly rare. Consequently, direct injection of cancer cells into the tibia is most commonly performed, but is unable to reflect early events in metastasis and is thus not considered to be a true metastatic model⁶. This issue is further complicated by observations of species-specific differences in the prostate cancer response to murine and human osseous tissue⁷. Additionally, animal models are typically costly, time-consuming and generally low-throughput, hampering their application for drug screening purposes. In contrast, in vitro culture models allow for the use of human-origin cells, are more accessible and less technically challenging. Cell culture-based model systems have grown increasingly complex, incorporating heterocellular cultures to more accurately

represent native tissue, and even three-dimensional culture systems to better reflect spatial considerations⁸ and mimic critical systems in human-on-a-chip models⁹.

Our group and others have previously described the co-culture of endothelial and mesenchymal stem cells for the generation of vascularised bony constructs for applications in regenerative medicine¹⁰. Under appropriate conditions, endothelial cells in co-culture with stromal cell types spontaneously form prevascular networks¹¹, yielding "organotypic" microvascular niches that are suggested to represent the pre-metastatic site. Indeed, cancer cells cultured in engineered bone microenvironments have been shown to mimic *in vivo* observations¹², and are thus being studied for drug screening applications. To further develop this system, we set out to explore the use of fluorescent labelled cells to facilitate observations and data acquisition. Thus, the purpose of this study was to develop an accessible co-culture system for the systematic evaluation of cancer cell response to the pre-metastatic bone niche. Our results demonstrated that heterocellular cross-talk between endothelial cells and mesenchymal stem cells potentiate vasculogenesis and osteogenesis in a co-culture system. Subsequent application of prostate cancer cells showed the formation of cell clusters reminiscent of micrometastatic sites, and the use of fluorescent labels facilitate the observation of cancer cell proliferation and migration. This system may thus be used to evaluate anti-proliferative and anti-migratory effects of therapeutic drugs, as well as to further our understanding of tumour-stromal interactions in the metastatic bone niche.

2. Experimental Section

Cell Culture

Mesenchymal Stem Cells (MSC)

Human tissue collection for research purposes was approved by the Domain Specific Review Board of National Healthcare Group (DSRB-D-06-154), in compliance with international guidelines regarding the use of fetal tissue for research. In all cases, patients gave separate written consent for the use of the collected tissue. Fetal femurs were collected for isolation of Mesenchymal Stem Cells (MSC) after clinically-indicated termination of pregnancy as previously described¹³. Briefly, single-cell suspensions were prepared by flushing bone marrow cells from femurs using a 22-gauge needle, passed through a 70µm cell strainer (BD Falcon, USA) and plated on 100mm culture dishes (NUNC, USA) at 200,000 mononuclear cells/cm². Non-adherent cells were removed by initial media changes; remaining adherent spindle-shaped cells were recovered from the primary culture after 4 to 7 days. Cells were trypsinized at sub-confluence and re-plated at 10⁴ cells / cm². Thus obtained cells were termed "MSC". Cells were maintained in Dulbecco modified Eagle media (DMEM)-Glutamax (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS), 50U/mL penicillin and streptomycin (GIBCO, USA; hereafter referred to as D10 media). Cells from passage 2-4 were characterized as previously described and used at passage 8 for subsequent experiments¹⁴.

Endothelial Cells (EC)

Human Umbilical Vein Endothelial Cells (HUVEC) were isolated using standard techniques as described by Baudin et al¹⁵. Briefly, human umbilical cords obtained from term deliveries rinsed and flushed through with PBS. The umbilical vein was identified and 0.2% collagenase solution was injected to detach endothelial cells. HUVEC were collected and plated on collagen coated plates in low-serum EGM (Lonza, Singapore). Fresh medium change was performed every 2 to 3 days, and cells were used in passage four through six for these experiments.

Prostate Cancer Cell line (PC3)

PC3, a highly aggressive cell line initiated from bone metastasis of a grade IV prostatic adenocarcinoma, were purchased from American Type Culture Collection (ATCC). Cells were maintained in RPMI growth media, comprising of RPMI media (Gibco), 10% FBS and 50U/mL penicillin and streptomycin. In all experiments, cells from passage 18–30 were used.

Lentiviral labeling

EC and PC3 were labelled with green fluorescent and histone 2B-red fluorescent fusion protein respectively using lentiviruses as previously described¹⁶. Briefly, for the generation of lentiviral vectors, transfer plasmids (Addgene; pRRLSIN.cPPT.PGK-GFP.WPRE and pHIV-H2BmRFP respectively) were co-transfected with pMD2.G and pCMV.ΔR8.74 into HEK293T cells. The supernatant was collected 2 and 3 days following transfection and concentrated by two rounds of ultracentrifugation at 50,000 g for 2 h and the final pellet was dissolved in a small volume of 1% BSA in PBS (1/100 of starting volume). The number of transducing units (TUs) of the vectors were determined by infecting 100, 000 293T cells using a serial dilution of the vector. The dilution resulting in <30% fluorescent cells was used to calculate the number of TUs per ml. For transduction, cells were seeded at 0.5×10^4 cells/cm² in T-25 flasks, and exposed to multiplicity of infection (MOI) of 5 of lentivirus with 4 mg/mL polybrene.

Immunocytochemistry

Cells were seeded at 4K cells per 100 µl into each well of 8 Well Permanox™ slide (System 177445; nunc brand of Thermo Scientific) for characterisation. Samples were washed in Phosphate Buffered Saline (PBS), fixed in a 1:1 methanol:acetone solution, washed twice with PBS and incubated in a blocking solution (10%BSA/Goat Serum/0.3%Triton-X) for 1 hour at room temperature. Staining was performed by addition of primary antibodies (MSC panel: CD 34, CD 73, and CD 105 from Miltenyi Biotech; CD 90 from Chemicon Europe; CD 45 from

BioLegend) (EC panel: CD 31 from Miltenyi Biotech; VEGFR2 and CD 144 from abcam; von Willebrand Factor from Dako) with a dilution factor of 1:100 and incubated for 1 hour at room temperature. Two washes were performed prior to incubation with secondary antibody (Chemicon International) at a ratio of 1:500. Slides were mounted in VECTASHIELD® mounting medium with DAPI and cells were viewed using confocal laser scanning microscopy (Olympus FV1000).

Osteogenic induction

Bone media (BM) was prepared using D10 medium supplemented with bone inducing elements - 10 mM β -glycerophosphate, 10^{-8} M Dexamethasone and 0.2 mM Ascorbic acid (Sigma Aldrich, USA). Osteogenic induction was performed by replacing maintenance medium with BM.

Osteogenic assays

Alizarin Red S (ARS) staining and quantification were performed as previously described¹⁴. Briefly, monolayers in 24-well plates well (n=3) were washed with phosphate buffered saline and fixed in 10% (v/v) formaldehyde (Sigma–Aldrich) at room temperature for 15 min. The samples were then washed twice with excess dH₂O prior to addition of 0.5 mL of 40 mM ARS solution (pH 4.2) per well. The plates were incubated at room temperature for 20 min. After aspiration of the unincorporated dye, the wells were washed four times dH₂O. ARS was extracted from the monolayer by incubation of the monolayers in 0.5 mL cetylpyridinium chloride (CPC; Sigma Aldrich) extraction buffer (10% w/v in 10 mM sodium phosphate buffer; pH 7.0) for 1 h. The dye was then removed and 200- μ L aliquots were transferred to a 96-well plate prior to reading at 570 nm.

Von Kossa staining was performed as previously described (8). Briefly, samples were gently rinsed twice with PBS then fixed with 10% formalin for 1 hour, followed by 2 washes with distilled water and stained with freshly made 2% Silver nitrate (Sigma Aldrich, USA) in distilled water (w/v) for 10 minutes in the dark and expose to sunlight for 30 minutes.

Vasculogenic induction

Cell suspensions of EC and MSC were obtained by trypsinisation, combined and inoculated on collagen-coated plates in EGM. Prevascular networks were formed spontaneously and allowed to proceed for 14 days. Cellular ratios were varied in a dose titration study to evaluate the effect of MSC on EC network formation. For conditioned media experiments, MSC were seeded to confluence and maintained in EGM for three days. Thus conditioned media was collected to study the influence of MSC-secreted soluble factors on EC network formation.

Vasculogenic assays

Figure 1. Quantification and assessment of vascular morphogenic parameters. (A) Predefined positions within a 24 well plate were marked out using a mask at the start of experiments (B) Fluorescent images of endothelial networks were obtained (C) Skeletal traces were obtained using CellProfiler software, from which (D) vascular morphometric data could be obtained.

Pre-defined positions were marked out using a mask as shown in Figure 1A. GFP-labelled EC were monitored using fluorescent microscopy and representative images were taken (Figure 1B).

Cell-profiler software was used to obtain skeletal outlines (Figure 1C), from which vascular morphometric parameters¹⁷ could be derived with a customised pipeline (Figure 1D).

Generation of triple cultures

40,000 EC and 40,000 MSC were added to each well in a 24 well plate and maintained in EGM to allow network formation. After 14 days, 800 PC3 cells were added to each well and maintained either in BM or EGM. Routine monitoring was performed by fluorescent microscopy.

PC3 Proliferation assays

Pre-defined positions were marked out using a mask as shown in Figure 1A. RFP-labelled PC3 cell nuclei were monitored using fluorescent microscopy and representative images were taken at 4x magnification. NIH ImageJ software was used to enumerate cells. Briefly, images were thresholded to yield binary images, inverted, and subsequently subject to particle analysis.

Statistical Analysis

All statistical analyses were carried out using Student's t test.

3. Results

Characterisation of cells

Figure 2. Characterisation of isolated cells. MSC were found to express stromal markers (A) CD73, (B) CD90 and (C) CD105, while EC continued to express CD31, VEGFR2 and vWF, confirming endothelial identity, even after lentiviral marking.

Isolated MSC were characterised prior to use in experiments. Consistent with current accepted guidelines, MSC were found express stromal markers (CD73, CD90 and CD105), but not haemopoietic markers (CD45, CD34; data not shown), indicating a mesenchymal stromal phenotype¹⁸. Correspondingly, EC continued to express mature endothelial markers (CD31, VEGFR2 and vWF), even after lentiviral marking, indicating the suitability for use in subsequent experiments.

Evidence for heterocellular interactions between EC and MSC

Upon induction, MSC demonstrate ability to undergo osteogenic differentiation, as evidenced by Alizarin Red S stain uptake (Figure 3A), as well as von Kossa staining (Figure 3B). In contrast, no mineral deposits were found when MSC cultured in both D10 and EGM maintenance medium (Figure 3C and D). When EC were cultured together with MSC in BM, increased ARS staining was observed (1.52 ± 0.23 mM vs 1.35 ± 0.16 mM), suggesting osteopotentiating influences by EC on the MSC.

Figure 3. Osteogenic differentiation assays. EC-MSC co-cultures were able to undergo osteogenic differentiation to deposit minerals, as evidenced by (A) Alizarin Red S and (B) vonKossa. (C, D) Similar staining could be observed on MSC monocultures, albeit at lower levels. In contrast, (E, F) MSC cultured in maintenance medium (D10) failed to demonstrate

appreciable mineralisation. (G) Alizarin red stain was eluted and quantified, showing elevated mineral deposition in the EC-MSC co-culture group. *** denotes a p-value of less than 0.001 when compared against the control MSC in D10 group.

Aside from the osteopotentiating effects of EC on MSC, reciprocal interactions could be seen, leading to vasculogenic events. When cultured with MSC, EC undergo network formation in a process akin to developmental vasculogenesis (Figure 4A), aggregating to form clusters within six days of inoculation, followed by sprouting and subsequent consolidation to form networked structures. In contrast, this process was not observed when EC were cultured in MSC-conditioned medium even after 21 days, suggesting cross-talk or direct cell-cell contact to be critical for the formation of such structures. This process was tracked and quantified using the CellProfiler software (Figure 4B, C and D), a free open-source software designed to quantitatively measure phenotypes automatically. A gradual increase in network complexity was evidenced by increasing tube length, which tended to regress by day 14. Similar trends were observed with branchpoint counts and segment counts. Dose titration suggest that network structures could be generated in a variety of EC:MSC ratios. When EC were added with MSC at 10:1 ratio, network formation was observed as early as day four post-seeding. However, the networks were not stable, and enter regression by day 7, suggesting a minimum threshold proportion of MSC is required to maintain stable networks, and providing further evidence for heterocellular communication between the two cell populations.

(A)

(B)

(C)

(D)

Figure 4. Vasculogenic effects of MSC on EC. (A) Direct co-cultures of EC with MSC result in vasculogenesis as demonstrated by the gradual formation of EC networks over fourteen days. Control shows EC maintained in MSC-conditioned media, with no networks generated, suggesting reciprocal cross-talk to be required for the vasculogenic effects. (C - D) Morphometric analysis using CellProfiler software shows vascular morphology to be dependent on cell ratio, with earlier tube and branchpoint formation when endothelial cells were seeded with MSC at at 10:1 ratio. These networks quickly regressed, however, suggesting a minimum proportion of MSC is required to stabilise the networks.

Inoculation of organotypic cultures with PC3 cells

EC-MSC cocultures were generated and mature networks were allowed to form over 14 days and PC3 cells were subsequently added to the resultant system . High resolution images could be obtained, allowing the visualisation of individual nuclei in the H2B.RFP-labelled PC3 cells to facilitate counting and / or tracking of migration. Additionally, it was observed that the signal intensities were sufficient to allow single cell monitoring even with a low-powered (4x) objective. Images taken three days post seeding show the emergence of tight clusters of PC3 cells sparsely distributed throughout culture system (Figure5, indicated by yellow arrowheads). Additionally, quiescent cells were sporadically found to be singly adhered.

Figure 5. (A) Visualisation of H2B.RFP-labelled PC3 cells inoculated on EC-MSC co-cultures. Images were obtained by confocal laser scanning microscopy with a 10x objective and subsequently stitched to provide a general overview. RFP-labelled nuclei could be clearly discerned, facilitating monitoring and observation. Cell clusters could be found three days post-inoculation (yellow arrowheads). Inset frame (B) shows the magnified view of the same region depicting PC3 cell clusters.

Proliferation of PC3 cells on simulated bone microenvironment.

To investigate the response of prostate cancer cells to the simulated bone environment, prostate cancer PC3 cells were seeded to the EC-MSC co-cultures. Multiple images were captured for each well (Figure 5A) and nuclei were enumerated (Figure 5B). Across all groups, proliferation of PC3 cells was initially slow due to the low density of seeding. Following the establishment of colonies from day 5, PC3 monocultures began to undergo exponential proliferation. In contrast, when seeded on MSCs, PC3 cells maintained a largely dormant state and showed no appreciable signs of proliferation, even in the absence of osteogenic induction. Finally, in the PC3, MSC and

EC triple co-culture group, PC3 proliferation rate was observed to be higher than that in the PC3-
MSC groups, with faster proliferation in the EGM group.

(A)

(B)

PC3 proliferation data

Figure 6. Proliferation of PC3 cells in simulate bone microenvironments. (A) Representative images of PC3 nuclei in a range of culture conditions. (B) From these images, PC3 cells could be easily enumerated, allowing longitudinal follow-up and assessment of proliferation or migration. Nuclei counting was automatically performed using ImageJ software. Results indicate altered cell proliferation rates in response to the substrate, with PC3 cell growth notably suppressed when cultured on MSC.

4. Discussion

Bone metastasis research is currently heavily dependent on animal model¹⁹. Such models are typically technically challenging, and face several limitations in reproducing species specific-responses. While humanised rodent models show promising results^{7, 20}, these systems present significant technical difficulties and are typically too expensive for drug screening purposes. In this study, heterogeneous cell cultures, which are currently being studied for regenerative applications^{10b, 21}, were adapted to simulate a bone microenvironment. PC3 prostate cancer cells were added, and monitoring of live cells was performed to study bone-cancer cell interactions. The model described here allows for ease of automation and scaling to facilitate drug screening. Additionally, the combined use of vascular and osteogenic cells create a more organotypic environment that better recapitulates the in vivo microenvironment²².

The link between prostate cancer and the osteogenic pathway is well-established²³, with multiple interactions on the endothelin (ET) axis²⁴. Osteoblasts and endothelial cells within the pre-metastatic bone site are thought to secrete various cytokines, including interleukin-1, triggering upregulation of ET-1 in metastatic prostate cancer cells, completing the paracrine loop

and leading to increased osteoblastic activity²⁵. In line with previous findings, we have found endothelial cells to be potentiate osteoblastic activity, suggesting a possible role of endothelial cells in potentiating tumour-induced new bone formation²⁶, and providing further support for the use of heterocellular cultures in this model.

In addition to the osteogenic influence of EC on MSC, reciprocal effects promoting vasculogenesis were observed in our culture system. Indeed, bone marrow-derived MSC have been shown to home to and stabilise microvasculature in vivo²⁷. Our results show MSC to have at least two major roles in the process of vasculogenesis in vitro: initiation of network formation and stabilisation of networks. Cross-talk and direct cell contact were demonstrated to be required for these influences, with conditioned media alone ineffective in stimulating vasculogenesis. Given the central role of endothelial cells in cancer biology, and in light of recent data suggesting the role of the perivascular niche in governing tumour dormancy²², stroma-mediated effects on vasculogenesis may provide an additional facet to understanding cancer progression. Thus, taken, together with the osteopotentiating effects of EC, three-way interactions between MSC, EC and prostate cancer cells may be central in governing cancer cell behaviour in the metastatic site. In developing this model, however, it was noted that although culture in osteogenic induction medium was able to elicit mineral deposition, it also resulted in reduced endothelial cell viability and deterioration of the endothelial network structures. Further work and optimisation remains to be done in order to generate a true vascularised bone environment. Here, we proceeded instead to conduct subsequent experiments separately in both pure bone induction medium and endothelial growth medium.

To study the effect of the simulated bone environment on prostate cancer cell proliferation, prostate cancer PC3 cells were added to EC-MSC co-cultures. h2B.RFP-labels were added to

facilitate visualisation and enumeration of the cells. Additionally, EC were labeled with GFP to enable observation of the prevascular networks. We have also demonstrated the use of image analysis software that may be useful for the evaluation of changes in vascular morphology as a result of interactions with the cancer cell. Here, we conducted concurrent observations of PC3 cells and EC in live cultures, observing the attachment of PC3 cells to the bone stromal environment, and also the formation of cell clusters. A major advantage of two dimension, in vitro systems is the accessibility for imaging. Here, imaging could be performed at a single cell level, and the capabilities may be further enhanced through the rational use of gene reporters²⁸ or other molecular imaging systems²⁹ that can provide real-time read-outs of the biological state of cells in situ.

To demonstrate utility for drug screening applications, proliferation capacities of PC3 cells on various substrates was evaluated. The cells were intentionally seeded at low densities to represent the low frequencies of metastatic cell arrest in vivo. Following an initial lag phase, PC3 cell proliferation was found to proceed exponentially in both BM and EGM. Of interest, it has previously been observed that prostate cancer cell proliferation on standard tissue culture surfaces far exceed in vivo growth rates, raising questions on the validity of the use of monocultures for drug screening³⁰. Conversely, when cultured on MSC monolayers, PC3 cell proliferation and migration were extremely low over 14 days, in both BM and EGM. The cause of this lack of proliferation is unclear, and is in sharp contrast to observations that stromal cells provide a supportive environment for breast cancer cell³¹. It may, however, reflect the latency period that is commonly observed in prostate cancer metastasis, following dissemination of tumour cells to the skeleton, and merits further investigation on this apparent dormancy. Finally, it was observed that, while maintenance in BM may be favorable to simulate osteogenic

conditions, it was also noted that EC could not be sustained for extended periods in BM alone, possibly accounting for the difference in PC3 proliferative potential within the triple co-cultures. Further optimization on appropriate media composition to reflect the in vivo environment is required. Additionally, in light of evidence suggesting the critical role of three-dimensional cultures in bone metastasis^{8, 12}, further work is underway to evaluate the performance of this system with engineered bone constructs¹⁰.

In summary, a co-culture system involving the use of EC, MSC and prostate cancer cells was developed as an organotypic model of the metastatic bone microenvironment. Osteogenic and vasculogenic processes were found to be influenced by the presence of co-cultures. PC3 cells were successfully seeded on this simulated bone stromal environment, and methods to automatically monitor and access vascular morphology and cell proliferation were discussed. This co-culture system represent a cheap and accessible method to study bone metastasis in vivo, which may be upscaled for automation.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

BM, Bone medium; D10, MSC maintenance medium; EC, Endothelial Cell; MSC, Mesenchymal Stem Cell; EGM, Endothelial Growth Medium

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