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A bioengineered microenvironment to quantitatively measure the tumorigenic properties of cancer-associated fibroblasts in human prostate cancer

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ABSTRACT

Stromal–epithelial cell interactions play an important role in cancer and the tumor stroma is regarded as a therapeutic target. *In vivo* xenografting is commonly used to study cellular interactions not mimicked or quantified in conventional 2D culture systems. To interrogate the effects of tumor stroma (cancer-associated fibroblasts or CAFs) on epithelia, we created a bioengineered microenvironment using human prostatic tissues. Patient-matched CAFs and non-malignant prostatic fibroblasts (NPFs) from men with moderate (Gleason 7) and aggressive (Gleason 8–9 or castrate-resistant) prostate cancer were cultured with non-tumorigenic BPH-1 epithelial cells. Changes in the morphology, motility and phenotype of BPH-1 cells in response to CAFs and NPFs were analyzed using immunofluorescence and quantitative cell morphometric analyses. The matrix protein gene expression of CAFs, with proven tumorigenicity *in vivo*, had a significantly different gene expression profile of matrix proteins compared to patient matched NPFs. In co-culture with CAFs (but not NPFs), BPH-1 cells had a more invasive, elongated phenotype with increased motility and a more directed pattern of cell migration. CAFs from more aggressive tumors (Gleason 8–9 or CRPC) were not quantitatively different to moderate grade CAFs. Overall, our bioengineered microenvironment provides a novel 3D *in vitro* platform to systematically investigate the effects of tumor stroma on prostate cancer progression.

1. Introduction

The cell microenvironment through multiple controlled signals directs fundamental cellular processes such as migration, proliferation, survival, and differentiation. Aberrant cues can result in diseases, such as cancer [1,2]. Specifically, in prostate cancer, the stromal microenvironment is critical in determining epithelial cell differentiation and function [3]. In tumor stroma, carcinoma-associated fibroblasts (CAFs) have a distinct phenotype relative to normal prostate fibroblasts (NPFs) and confer tumorgenicity on non-tumorigenic prostatic epithelial (BPH-1) cells when xenografted *in vivo* [4–7]. Based on these observations, CAFs are a therapeutic target.

At present, the only proven method of investigating CAF tumorigenicity is tissue recombination, where stroma and epithelia are co-grafted into immune-deficient mice and the outcome is whether or not a tumor is formed. We and others have widely used

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this bioassay, yet the techniques are lengthy and technically challenging; only recently we developed a method for unbiased semi-quantitative analyses [4]. To advance the field, there is a need to develop improved *in vitro* models to mimic the cancer cell interactions with the microenvironment and allow quantitative analyses of the outcome, in terms of their effect on the epithelium. In turn, this will enable the comparison of relative differences between different patient CAFs. Ultimately, this data is fundamental to identify mechanisms that underpin therapeutic targeting of tumor stroma.

In the past, *in vitro* models of cancer cells have largely depended on the use of two-dimensional (2D) tissue culture on plastic or glass surfaces. Although much has been learned from these studies about how paracrine mechanisms instruct or direct cell behavior, these approaches are limited by the fact that the cells on surfaces often experience artificial polar environments, atypical elastic properties of their environment, and non-physiological cell contacts and densities. Moreover, the flat surface of the tissue culture plate represents a poor topological approximation of the more complex three-dimensional (3D) architecture of the extracellular matrix (ECM) [8]. Therefore, more physiologically mimetic model systems to study both normal and abnormal functions of cells and tissues are desirable [9]. There is strong evidence exists that the highly porous nano- and microtopography that results from the 3D fibrillar associations of ECM proteins is essential for cell adherence, cytoskeletal organization, migration, signal transduction, morphogenesis and differentiation in cell culture [10,11].

The hypothesis of this study was that production of ECM components in a cellularized co-culture method would allow *in vitro* validation of prostatic CAFs. Herein, a bioengineered approach was developed to compare the effects of human primary stromal fibroblasts on epithelial cell morphology and motility and test if there is a change with prostate cancer progression.

2. Materials and methods

2.1. Isolation of primary prostatic stromal cells (CAFs and NPFs)

Human prostate specimens were obtained by the Australian Prostate Cancer BioResource with approval from the Cabrini Institute (03-14-04-08), Epworth Hospital (34306) and Monash University Human Research Ethics Committees (2004/145). Tissue was collected from radical prostatectomy specimens for CAFs and NPFs as previously described [4]. Transurethral resection of the prostate (TURP) tissue was used for benign prostate fibroblasts (BAFs) and CAFs from castrate resistant prostate cancer (CRPC). Prostate tissue specimens were diced into 2–3 mm^3 pieces, then digested in digestion media (RPMI, 10% fetal calf serum (FCS), 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 μg/mL fungizone, 100 μg/mL gentamicin, 225 U/mL Collagenase Type I (Sigma–Aldrich) and 125 U/mL Hyaluronidase Type II (Sigma–Aldrich)) for approximately 16 h. Cell suspensions were washed with PBS and transferred into T75 flasks with RPMI (School of Biomedical Sciences, Media and Prep Services, Monash University) containing 5% heat inactivated FCS (ThermoScientific), 1 nM testosterone (Sigma–Aldrich), 10 ng/mL fibroblast growth factor (FGF; Millipore), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco), which allows for the selective growth of stromal cells. Cells were maintained at 37 °C in 5% CO_2 atmosphere. Matched stromal cells from five patients and unmatched CAFs from two patients with CRPC were used for this study between passages 2 and 10. For all 7 patients (i–vii), the original patient ID and pathology are recorded in Table 1.

2.2. BPH-1 cells

BPH-1 cells [12] were grown in RPMI media, supplemented with 5% heat inactivated FCS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2.

2.3. Animals

All animal handling techniques and procedures were conducted in accordance with National Health and Medical Research Council guidelines for the care and use of Laboratory Animal Act according to the Animal Experimentation Ethics Committee at Monash University (Approval Numbers: MMCA/2008/33). Sprague Dawley day 0–1 rats were obtained from Monash University Central Animal Services (Clayton, Australia) and culled. Seminal vesicles (SVs) were dissected in DMEM F-12. The mesenchyme (SVM) was dissociated from the epithelium by digestion in 1% trypsin for 70 min and separated into mesenchyme and epithelial components by dissection. SVM were transferred to fresh DMEM with 20% FCS where they were stored on ice until recombinant grafts were made. Non-obese diabetic severe combined immune-deficient (Nod-SCID) 6–8 weeks old male mice were obtained from Animal Resources Centre (Canningvale, Australia) for grafting of recombinants.

Table 1
Primary prostatic stromal cell lines used in study.

		Patient	Cell line	Pathology (Gleason score)
Gleason 7	i	26	CAF	3 + 4 (7)
			NPF	Benign
	ii	35	CAF	4 + 3 (7)
			NPF	Benign
	iii	72	CAF	3 + 4 (7)
			NPF	Benign
Gleason 8–9	iv	62	CAF	4 + 5 (9)
			NPF	Benign
	v	64	CAF	4 + 4 (8)
			NPF	Benign
CRPC	vi	CRPC − 4	CRPC-CAF	4 + 5 (9)
	vii	CRPC − 5	CRPC-CAF	4 + 5 (9)

CPRC − castrate-resistant prostate cancer.

2.4. Tissue recombination

Tissue recombination involves mixing of stromal and epithelial cell populations which are combined and then grafted into immune-deficient host mice, as previously described [7,13]. For this study tissue recombinants were generated from NPFs, CAFs, BAFs or SVM (~ 2.5×10^5 cells), mixed with 1×10^5 BPH-1 cells. Recombinants were embedded in 10–50 μl of type I rat tail collagen and incubated overnight in a 5% CO_2 humidified incubator at 37 °C in complete RPMI 1640 with 1 nM testosterone. The following day, recombinants were surgically implanted under the renal capsule of Nod-SCID mice, typically with 3–4 grafts on each kidney. Nod-SCID hosts were supplemented with 5 mm sub-cutaneous testosterone (T) implants. Recombinant tissues were grown in host mice for 8 weeks. At the time of harvest, wet weights of tissue recombinants were recorded and tissues were fixed in 10% formalin and embedded in paraffin wax for histological analysis by hematoxylin and eosin staining.

2.5. In vitro co-culture

CAFs or NPFs were seeded at 3×10^3 cells/cm^2 onto *Thermanox*™ coverslips (∅ 13 mm, ThermoScientific) in 24 well plates (BD Falcon). Cells were cultured at 37 °C, 5% CO_2 and after three days the medium was supplemented with 50 μg/mL ascorbic acid (Sigma) to stimulate extracellular matrix deposition [14]. Fibroblasts were cultured for a further 2 weeks to yield a dense monolayer with extensive ECM deposition. Then 1.5×10^4 BPH-1 cells pre-stained with Cell Tracker green CMFDA (5-chloromethylfluorescein diacetate; Invitrogen Molecular Probes, USA) were seeded on top of the fibroblasts and cultured at 37 °C, 5% CO_2 for 3 days.

2.6. Immunocytochemistry

After 2–3 passages, stromal cells were trypsinized and 5×10^3 cells were seeded into wells of Millicell EZ slides (Merck Millipore, Massachusetts, USA) and grown overnight in a 37 °C, 5% CO_2 humidified incubator. Cells were stained for vimentin (Sigma–Aldrich, Missouri, USA; 5 μg/mL) and smooth muscle actin (Sigma–Aldrich; 1 μg/mL). Epithelial specific markers such as cytokeratin 8/18 (Leica Biosystems, Germany; 2.1 μg/mL) and high molecular weight cytokeratin (CKHMW; Dako, Denmark; 0.18 μg/mL) were used to identify any contaminating non-stromal cells. Staining was performed as previously reported [15]. Briefly, cells were fixed in 4% formalin, permeabilized with 0.3% Triton X-100, and blocked using peroxidase and CAS blocks prior to incubation with primary antibodies or matching isotype controls. Staining was visualized using anti-mouse polymer-HRP and ABC (Dako), followed by DAB+ chromagen solution for 3–5 min and hematoxylin counterstaining.

2.7. Immunofluorescence

Samples were fixed with 4% formaldehyde (Sigma–Aldrich) for 10 min at room temperature, washed twice with PBS and then permeablized for 5 min with 0.2% Triton X (BDH, Poole, UK)/PBS. After a 10 min blocking step with 2% BSA (Sigma)/PBS, samples were incubated with 10 μg/mL of mouse anti-human fibronectin (HFN 7.1, DSHB), in 2% BSA in PBS for 1 hour at room temperature; mouse IgG (Invitrogen) was used as a control. After washing with PBS, samples were incubated with 10 μg/mL fluorescently labeled secondary antibody (cell signaling, anti-mouse Alexa 633), 8 U/mL phalloidin-TRITC (Invitrogen), and 5 μg/mL DAPI (Invitrogen) diluted in 2% BSA in