Development of a 3D culture system to study the skeletal metastasis of prostate cancer

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3D model, 3D culture, hydrogel, matrix, tissue engineering, prostate cancer, LNCaP, human osteoblast, bone metastasis, co-culture
Abstract

In the cancer research field, most in vitro studies still rely on two-dimensional (2D) cultures. However, the trend is rapidly shifting towards using a three-dimensional (3D) culture system. This is because 3D models better recapitulate the microenvironment of cells, and therefore, yield cellular and molecular responses that more accurately describe the pathophysiology of cancer. By adopting technology platforms established by the tissue engineering discipline, it is now possible to grow cancer cells in extracellular matrix (ECM)-like environments and dictate the biophysical and biochemical properties of the matrix. In addition, 3D models can be modified to recapitulate different stages of cancer progression for instance from the initial development of tumor to metastasis. Inevitably, to recapitulate a heterotypic condition, comprising more than one cell type, it requires a more complex 3D model. To date, 3D models that are available for studying the prostate cancer (CaP)-bone interactions are still lacking. Therefore, the aim of this study is to establish a co-culture model that allows investigation of direct and indirect CaP-bone interactions. Prior to that, 3D polyethylene glycol (PEG)-based hydrogel cultures for CaP cells were first developed and growth conditions were optimised. Characterization of the 3D hydrogel cultures show that LNCaP cells form a multicellular mass that resembles avascular tumor. In comparison to 2D cultures, besides the difference in cell morphology, the response of LNCaP cells to the androgen analogue (R1881) stimulation is different compared to the cells in 2D cultures. This discrepancy between 2D and 3D cultures is likely associated with the cell-cell contact, density and ligand-receptor interactions. Following the 3D monoculture study, a 3D direct co-culture model of CaP cells and the human tissue engineered bone (hTEBC) construct was developed. Interactions between the CaP cells and human osteoblasts (hOBs) resulted in elevation of Matrix Metalloproteinase 9 (MMP9) for PC-3 cells and Prostate Specific Antigen (PSA) for LNCaP cells. To further investigate the paracrine interaction of CaP cells and (hOBs), a 3D indirect co-culture model was developed, where LNCaP cells embedded within PEG hydrogels were co-cultured with hTEBC. It was found that the
cellular changes observed reflect the early event of CaP colonizing the bone site. In the absence of androgens, interestingly, up-regulation of PSA and other kallikreins is also detected in the co-culture compared to the LNCaP monoculture. This non androgenic stimulation could be triggered by the soluble factors secreted by the hOB such as Interleukin-6. There are also decrease in alkaline phosphatase (ALP) activity and down-regulation of genes of the hOB when co-cultured with LNCaP cells that have not been previously described. These genes include transforming growth factor β1 (TGFβ1), osteocalcin and Vimentin. However, no changes to epithelial markers (e.g E-cadherin, Cytokeratin 8) were observed in both cell types from the co-culture. Some of these intriguing changes observed in the co-cultures that had not been previously described have enriched the basic knowledge of the CaP cell-bone interaction. From this study, we have shown evidence of the feasibility and versatility of our established 3D models. These models can be adapted to test various hypotheses for studies pertaining to underlying mechanisms of bone metastasis and could provide a vehicle for anticancer drug screening purposes in the future.
List of publications


List of conference abstracts and presentations


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4.2 Materials and methods
4.3 Results
4.4 Discussion

Chapter 5: Utilization of a two-construct 3D in vitro model to study the paracrine interaction of prostate cancer (CaP) cells and human osteoblasts (hOBs)

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List of abbreviations

2D  2-dimensional
3D  3-dimensional
ADT  Androgen deprivation therapy
AFM  Atomic force microscopy
ALP  Alkaline phosphatase
AR  Androgen receptor
BC  Breast cancer
BM  Bone marrow
BMP  Bone morphogenetic proteins
BMSC  Bone marrow stromal cells
BSA  Bovine serum albumin
BSP  Bone sialoprotein
CAM  Cell adhesion molecules
CaP  Prostate cancer
CK  Cytokeratin
CLSM  Confocal laser scanning microscopy
CM  Conditioned media
CXCL12  Chemokine ligand 12
CXCR4  Chemokine receptor type 4
CYP11A1  Cytochrome P450, family 11, subfamily A, polypeptide 1
DAPI  4',6-diamidino-2-phenylindole
DHT  Dihydrotestosterone
Ecad  E-cadherin
ECM  Extracellular matrix
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
EMT  Epithelial- mesenchymal transition
ET-1  Endothelin-1
FASN  Fatty acid synthase
FBS  Fetal bovine serum
FDA  Fluorescein diacetate
FGF  Fibroblast growth factor
FITC  Fluorescein isothiocyanate
GF  Growth factor
H&E  Haematoxylin and eosin
HA  Hydroxyapatite
HCl  Hydrochloric acid
HMDS  Hexamethyldisilazane
hOB  Human osteoblast
HSD17B3  Hydroxysteroid (17-beta) dehydrogenase 3
hTEBC  Human tissue engineered bone construct
IGF  Insulin growth factor
IL  Interleukin
KLK  Kallikrein
KRT  Keratin
MMP  Matrix metalloproteinase
mPCL-TCP  Medical grade polycaprolactone-tricalcium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>phosphate</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>OCM</td>
<td>hOB conditioned media</td>
</tr>
<tr>
<td>OCN</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OT</td>
<td>Osteoblast-derived tissue</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethelene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PIA</td>
<td>Proliferative inflammation atrophy</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PLG</td>
<td>Poly (lactide-coglycolide)</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PSCA</td>
<td>Prostate stem cell antigen</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa-B</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RDH5</td>
<td>Retinol dehydrogenase 5</td>
</tr>
<tr>
<td>RWV</td>
<td>Rotating wall vessel</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Td</td>
<td>Doubling time</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue engineering</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plaminogen activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Statement of original authorship

The work contained within this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature ............................

Date   ..............................
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Chapter 1: Introduction
Chapter 1: Introduction

The staggering incidence of prostate cancer (CaP) in North America, Europe and Australia throughout this 21st century is a growing concern for these countries. In Australia alone, it is predicted that one in nine men will suffer from CaP in their lifetime (Prostate Cancer Foundation annual report 2011). Worldwide, this disease is the second leading cause of cancer death in men. While localised tumor is curable, CaP that has metastasised to secondary sites significantly reduces the survival of patients. The dominant metastatic site is the bone and usually bone lesions are presented as increased in abnormal bone formation (osteoblastic lesions). These osteoblastic lesions lead to bone fracture, spinal compression and severe morbidity to patients. To date, there is still no cure for bone metastasis and only palliative treatments are available. In general, 70-80% of bone metastasis cases occur in advanced CaP patients, with less than 35% survival within 5 years [1]. Bone metastasis is a multistage process that involves local invasion in the prostate, then, distant invasion via intravasation, circulation in the vascular or lymphatic system, extravasation and finally colonization in the new site. However, little is known regarding the mechanisms involved in cancer cell homing and establishment of micrometastases in the bone. A more detailed explanation of the pathophysiology of CaP and bone metastasis is provided in chapter 2. Due to the complexity of the development and progression of CaP, there is still a body of research to be explored to increase our understanding of the cancer biology. This requires in vitro and in vivo models that can represent a certain stage of the disease to drive such investigative studies. A better in vitro system that could more accurately translate the results for clinical applications compared to the traditional two-dimensional (2D) cultures is greatly needed. Therefore, the main aim for this PhD project is to develop a 3D in vitro model which allows studies of interactions between the CaP and bone cells and/or bone microenvironment.

Current models used for cancer research consist mainly of 2D cultures and mouse in vivo models. Although 2D based studies have greatly contributed to the fundamentals of cancer biology, it is undeniable that cells on flat surfaces are not representative of their native tissue where cells
interact with their three-dimensional (3D) microenvironment. The inadequacy of current in vitro models is very well reflected in the poor outcome of drug development and approval process [2]. 2D based assays clearly remain a poor predictor of antitumor drug in vivo efficacy. Hence, an alternative system using animal models can better mimic the physiological condition however, this system is complex and introduces many variables that could influence the outcome of an experiment. Such limitations from 2D cultures and in vivo models have lead to the development and utilization of 3D in vitro culture systems to bridge the gap between both systems. With the recent advances in the tissue engineering discipline, the technology and biomaterials are now available for the benefit of cancer research. The growing interest of using 3D cultures has availed opportunities to further explore tumor developmental biology which is not possible in 2D cultures. The relevance of using 3D cultures and the available 3D models for cancer research are discussed in more detail in chapter 2.

One of the well accepted forms of 3D tumor models, that represents the early non-vascular tumor development, is multicellular aggregates or, as sometime known, spheroids. Morphologically, the spheroid resembles avascular tumors with oxygen and nutrient gradients. There are a few methods that have been used to generate spheroids either by culturing cells in a condition that hinder cell attachment to surfaces or by embedding them in matrices [3-5]. The former approach utilizes liquid-overlay and rotating vessel wall methods, both methods promote spontaneous aggregation of cells to form spheroids. Matrix-embedding methods on the other hand, allow single cells to form a multicellular mass of various morphologies [6-8]. These matrices, in particular in the form of hydrogels render high viscoelasticity and diffusive transport that resemble natural extracellular matrix (ECM) [9]. In order to recapitulate the cell-matrix interaction and to correlate that to the cell behaviour, we had attempted to culture the CaP cell line LNCaP in hydrogels to form a 3D multicellular mass.

The current gold standard hydrogels used for culturing cancer cells in 3D are the basement membrane extract of mouse Engelbreth-Holm-Swarm sarcoma (Matrigel™) and collagen type I. Both hydrogels are derived from animal tissues and are very favourable for cell growth. However, the
biophysical and biochemical components of these natural hydrogels are not easily modified to tailor for specific requirements of an experiment or biological hypothesis. For example, in natural hydrogels their matrix stiffness cannot be independently altered without changing the protein density of the matrix. Moreover, natural hydrogels often present batch-to-batch variation that will introduce inconsistencies in results [9, 10]. Taken together the limitations of natural ECMs, a transition towards using synthetic hydrogels is an increasing trend seen in the literature. Synthetic hydrogels composed of inert materials such as polyethylene glycol (PEG) are used as the base material. This gives polymer chemists the opportunity to build in tuneable biochemical and biophysical properties as means to functionalise and control the hydrogel’s intrinsic factors. The utilization of synthetic hydrogels is still at its infancy in cancer research, thus the development and characterization of 3D culture models based on this type of biomaterials are rare. Hence, this PhD project’s initial focus is on the development and characterization of a 3D model system by using a biomimetic PEG hydrogel.

In chapter 3, phenotypic characterization of LNCaP cells cultured within PEG hydrogels in comparison to 2D cultures is described. The differences observed in 2D and 3D cultures involve in particular, the morphological changes and cell response to androgen stimulation. It is clearly shown that the LNCaP colonies formed in the hydrogels resemble the 3D architecture of avascular tumor with extensive cell-cell contacts that are clearly not present in 2D cultures. In addition, altered LNCaP cell response to stimulation with the synthetic androgen, R1881, is found in the 3D culture when compared to 2D cultures. The biochemical analyses also reveal that androgen receptor (AR) gene expression and protein synthesis from 2D cultures are not in consistent with 3D cultures. These discrepancies are also discussed in chapter 3. As seen from our results, 3D cultures are not only useful for studying early stage of CaP development but can also be adapted for more complex models such as the bone metastasis model.

Most studies of CaP mediated bone metastasis is restricted to animal models partly because the CaP cell and bone interaction is dynamic and the disease progression can only be best mimicked in *in vivo* models. The complexity of *in vivo* models often complicates the elucidation of specific
biological hypotheses, e.g. signalling pathways and/or cellular responses triggered by specific cell-cell or cell-matrix interactions. With 3D in vitro culture systems, these interactions are better defined and inconsistencies can be minimised as cell responses are not dependent on the entire host system. It is hypothesized in chapter 4, that tissue engineered bone (TEB) which was traditionally developed for cell-based therapy in bone disease/trauma is a potential tool for in vitro bone metastasis studies. Work over the last 10 years has shown that TEB can be reproducibly engineered. They have been tested in several in vivo models and in patients for its compatibility and ability to regenerate bone [11]. For this PhD project, the cell sheet-based methodology established by the Hutmacher group is utilised to fabricate a human tissue engineered bone construct (hTEBC) from primary human osteoblasts (hOBs). Adopting a similar method introduced by Zhou et al. (2007), the mineralised hOB sheet, which also consists of ECM proteins is used to wrap the medical grade polycaprolactone tricalcium phosphate (mPLC-TCP) scaffolds [12]. The hTEBC serves as a site for the initial stage of CaP cells encountering the bone/bone microenvironment. To depict the early stage of bone metastasis, two different approaches were adopted: either by direct or indirect co-culture of the CaP cell lines, LNCaP or PC3, with hTEBC. For direct co-cultures, both CaP cells and hOBs are in physical contact with each other. The establishment of this direct co-culture 3D model and its application for studying CaP-hOB interactions are further described in chapter 4. In this study, to our disadvantage, we experienced difficulty in separating both cell types for further analysis. This issue however can be resolved with an indirect co-culture model.

While most 3D bone metastasis models involve direct co-culture of both, cancer cells and osteoblasts, this approach sometimes does not fully mimic ECM that interacts with cells. ECMs which evidently govern cell behaviour including tumor progression are poorly integrated into previous models especially in suspension cultures. From our previously established direct co-culture model described in chapter 4, modifications were made to yield a more functional and versatile model in the form of indirect co-cultures. The importance of cell-matrix interactions are taken into account here, where both cell types, the LNCaP cells and hOBs, can receive cues from their
surrounding matrices. By approximating the mechanical and geometrical properties of the matrices, this gives rise to a more biologically relevant early stage bone metastasis model. In this indirect co-culture model, CaP cells that were encapsulated within the PEG hydrogel were not in contact with hOBs from hTEBC. To our advantage, it overcomes the difficulty of segregating both cell types for downstream analyses. In addition, this model allows examination of CaP progression under the influence of paracrine interaction between the CaP cells and hOBs. Characterization of the indirect co-culture model and assessment of this biphasic system for studying the indirect bi-directional interaction between CaP cells and hOBs is further described in chapter 5.

The final chapter summarizes the establishment of the 3D in vitro model used for studying cell morphogenesis and intercellular interactions between CaP cells and hOBs. It also highlights the feasibility of these models as tools for studying cellular and molecular changes in response to androgen induction.
Chapter 2: Literature Review
Chapter 2: Literature Review

2.1 Prostate Cancer

2.1.1 Background

In the United States and Canada it is predicted that prostate cancer (CaP) is the most diagnosed male cancer by 2010, accounting for at least 27% of all new cancer cases [13, 14]. This cancer is also prevalent in Australia being the most common male cancer. In Australia alone, CaP is the second leading cause of cancer death where almost 3 000 men die from this disease annually. A staggering 20 000 new cases are diagnosed each year making CaP the most common cancer in Australian men [15]. With early detection and treatment of CaP when the tumor is still localised, the survival rate in 5 years is 100%. Treatment selection for localised tumors depends on factors including the stage of cancer, serum Prostate specific antigen (PSA) level and general health of patients. To date, the underlying cause of CaP is still obscure. There are however risk factors associated with CaP that have been identified so far which are advancing age, family history, race and possibly diet [16].

2.1.2 Pathophysiology of CaP

A normal prostate gland consists of secretory luminal cells, neuroendocrine cells and basal cells. These compartments are separated from the stroma by a layer of basement membrane (Figure 2.1). The stroma consists of a mix cell population of smooth muscle, fibroblasts, vascular endothelial cells, nerve cells and inflammatory cells [17]. The development of normal to cancerous prostate cells is a slow process that arises from transformed prostate glands. These transformed glands appear as confined clumps among the normal tissue, known as carcinoma in situ or prostatic intraepithelial neoplasia (PIN). There are reports suggesting that proliferative inflammation atrophy (PIA) is associated with PIN. From histology examinations of human tissues, De marzo and others have observed a morphology transition between areas
Figure 2.1 Structure of normal prostate gland. (A) The prostate gland consists of luminal epithelium, neuroendocrine cells, a basal cell layer and a basement membrane. The basement membrane separates the gland from the surrounding stroma tissue. (B) Haematoxylin and eosin (H&E) histology examination of normal prostate gland. Bold arrow points to the intact basement membrane and thin arrow points to the basal cells underneath the luminal epithelium. Adapted from Liu et al. (2009) and Bok et al. (2002) [18] [19].

of PIA/proliferative atrophy and high grade PIN [20, 21] both often occur in the peripheral zone of the prostate [11]. Accumulation of genetic changes then further promotes cellular and molecular changes leading to high grade PIN. Low and high grade PIN are characterised by abnormal proliferation of cells within the prostatic ducts, ductules and large acini with cellular dysplasia. However, there is no invasion of the basement membrane.

High grade PIN is a premalignant state of prostate carcinoma and presents most of the morphological, biochemical and genotypic changes found in prostatic carcinoma [22, 23]. Some of the similarities between PIN and CaP are cellular proliferation within pre-existing ducts and acini, and enlargement of nucleus with a decrease in neuroendocrine cells. Unlike carcinoma of the prostate, which lacks the basal cell layer, it is still intact in PIN [22]. The premalignant cells then multiply to form larger tumors and subsequently become malignant. One of the hallmarks of CaP is the lack of the basal cell layer and in addition, the gain of a basal-like phenotype of the luminal epithelium. Although the epithelium still expresses the luminal marker Cytokeratin18 (CK18), simultaneously, expression of intermediate markers such as prostate stem cell antigen (PSCA) is also observed in the tissue [24-26]

Differentiation markers such as neutral mucin, prostatic acid phosphatase (PAP) and PSA are downregulated, while secretory proteins namely, estramustine binding protein, estrogen-inducible protein A-80, fatty
acid synthase and glycoprotein A-80 are upregulated in both CaP and PIN. PSA serum levels however, increase during CaP due to leakage of PSA from disrupted prostate glands into the vascular system [22, 27]. Apart from changes in cell morphology and protein expression, other changes at genotypic level also occur in CaP as listed in Table 2.1. Some of the genes and oncogene mutations that are involved in the alteration of CaP cell regulation are the ras and c-myc oncogenes, the bcl-2 proto-oncogene, p53, and retinoblastoma genes [28]. Genetic changes among other factors are also implicated in contributing towards malignant phenotype in CaP progression.

Table 2.1 Genetic and epigenetic changes in CaP. Adapted from De Marzo et al. (2007) [16].

<table>
<thead>
<tr>
<th>Gene and gene type</th>
<th>Location</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN1B Tumour-suppressor genes</td>
<td>12p13.1-p12</td>
<td>Encodes the cyclin-dependent kinase inhibitor p27. One allele is frequently deleted in primary tumours.</td>
</tr>
<tr>
<td>NXN1.1</td>
<td>8p21.2</td>
<td>Encodes prostate-restricted homeobox protein that can suppress the growth of prostate epithelial cells. One allele is frequently deleted in primary tumours.</td>
</tr>
<tr>
<td>PTEN</td>
<td>10q22.31</td>
<td>Encodes phosphatase and tensin homologue, which suppresses cell proliferation and increases apoptosis. One allele is frequently lost in primary tumours. Some mutations are found in primary tumours and more in metastatic lesions.</td>
</tr>
<tr>
<td>TP53</td>
<td>17p13.1</td>
<td>Has many tumour-suppressor functions, including cell-cycle arrest in response to DNA damage, senescence in response to telomere dysfunction, and the induction of apoptosis. Mutations are uncommon early, but occur in about 50% of advanced or hormone-refractory prostate cancers.</td>
</tr>
<tr>
<td>MYC Oncogenes</td>
<td>8q24</td>
<td>A transcription factor that regulates many target genes involved in cell proliferation, senescence, apoptosis and cell metabolism. Overexpression can directly transform cells. mRNA levels are commonly increased in all disease stages through unknown mechanism(s). Low-level amplification of the MYC locus is common in advanced disease.</td>
</tr>
<tr>
<td>ERG</td>
<td>21q22.3</td>
<td>Proposed new oncogene for prostate cancer. Fusion transcripts with the 5′ portion of androgen-regulated gene (TMPRSS2) arise from deletion or chromosomal rearrangements commonly found in all disease stages.</td>
</tr>
<tr>
<td>ETV1–4</td>
<td>7p21.3, 19q13.12, 1q11-q23, 17q11.31</td>
<td>Encodes ETS-like transcription factors 1–4, which are proposed to be new oncogenes for prostate cancer. Fusion transcripts with the 5′ portion of androgen-regulated gene (TMPRSS2) arise from chromosomal rearrangements commonly found in all disease stages.</td>
</tr>
<tr>
<td>Ar</td>
<td>Xq11–12</td>
<td>Encodes the androgen receptor. Protein is expressed in most prostate cancers, and the locus is amplified or mutated in advanced disease and hormone-refractory cancers.</td>
</tr>
<tr>
<td>Activation of the enzyme telomerase</td>
<td></td>
<td>Maintains telomere function and contributes to cell immortalization. Activated in most prostate cancers. Activation may be through MYC activation.</td>
</tr>
<tr>
<td>Caretaker genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTP1</td>
<td>11q13</td>
<td>Encodes the enzyme that catalyzes the conjugation of reduced glutathione to electrophilic substrates. Functions to detoxify carcinogens. It is inactivated in more than 90% of cancers by somatic hypermethylation of the Cpg island within the upstream regulatory region.</td>
</tr>
<tr>
<td>Telomere dysfunction</td>
<td>chromosome termini</td>
<td>Contributes to chromosomal instability. Shortened telomeres are found in more than 90% of prostate intraepithelial neoplasia (PIN) lesions and prostate cancer lesions.</td>
</tr>
<tr>
<td>Centrosome abnormalities</td>
<td>N/A</td>
<td>Contributes to chromosomal instability. Centrosomes are structurally and numerically abnormal in most prostate carcinomas.</td>
</tr>
<tr>
<td>Other somatic changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTGS2, APC, MDR2, DNA-B, RASSF1a, RARβ2</td>
<td>Various</td>
<td>The hypermethylation of Cpg islands within upstream regulatory regions occurs in most primary tumours and metastatic lesions. The functional significance of these changes is not yet known.</td>
</tr>
</tbody>
</table>
2.1.3 Dissemination of CaP cells

Metastasis occurs when malignant cells from primary sites spread and colonize distant sites. In summary, these complex multistage mechanisms involve local invasion, intravasation, dissemination via lymphatic or hematogenous system, extravasation and colonization (Figure 2.2) [29-31]. In order for cells to invade neighboring tissues or enter the existing/newly formed blood vessels, cells need to detach from the primary tumor site. This is accomplished when cell-cell contact is lost and cells become more motile or mesenchymal-like, often adopting an epithelial to mesenchymal transition (EMT). Simultaneously, the extracellular matrix is actively degraded by proteolytic enzymes such as kallikreins, matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) [32-34]. The proteolytic activity helps to break down tissue barrier (basement membrane and interstitial connective tissue) and allow cancer cells intravasation into the circulatory system [30]. In the vascular system, cancer cells that survive the brutality of the immune reaction and mechanical stress of rapid blood flow, eventually re-adhere to the blood vessel and extravasate. When cancer cells reach the new site, if condition permits cell survival, the cancer cell may remain viable. They then, either become dormant or progress to proliferate and colonize the new tissue site.

This newly established cancer colony is known as micrometastases [35, 36]. To successfully invade and home to a new tumor site, the cell would have to overcome all the cellular or physiological barriers to survive the host’s defence mechanisms. Among all prostate neighbouring organs, bone is the most prevalent metastatic site. However, the underlying cause for bone predilection is still not fully understood. From primary sites to secondary sites, numerous factors are involved in this progression. The multistage process is further explained below.
2.1.4 Bone specific metastasis

The bone environment, including the inorganic and organic components, is often associated with the progression of CaP to bone metastasis [38-40]. The mineralised tissue is defined by depositions of calcium and phosphate, predominantly hydroxyapatite (HA). Apart from the calcified tissue, organic components also contribute to the bone ECM. Such proteins provide both structural support (e.g. collagen, osteocalcin) and act as a storage for growth factors (GFs) namely Bone morphogenetic proteins (BMP), Transforming growth factor β (TGFβ) and Vascular endothelial growth factor (VEGF) [41, 42]. These minerals and biological components of the bone tissue are believed to play an important role in bone metastasis. In advanced CaP, bone metastasis often occurs at the axial skeleton regions particularly the trabecular bone and the proximal ends of the femur. CaP cells can metastasize to the region close to the prostate (e.g lumbosacral) and regions outside the spinal column and pelvis (e.g. skull and ribs). This is due to the rich venous drainage through Batson’s plexus that allows circulation of cancer cells. Although the predilection of CaP cells to bone is not fully
understood, the general understanding is that homing of CaP cells to bone is due to:

a) Cell migration driven by chemoattractants
b) Preferential arrest and attachment of circulating cells to endothelial cells of receptive organs
c) Favourable environment that is conducive for cancer growth and establishment of micrometastases after extravasation

According to an in vivo study by Tsingotjidou et al. (2001), CaP cells infiltrate the human adult bone implanted in Severe Combined Immunodeficiency (SCID) mice rather than the host bone [43]. This suggests that the human bone derived factors could play a role in attracting circulating CaP cells. In addition, in vitro assay also confirms that PC-3 cells and DU145 CaP cells preferentially migrate towards bone extract [44]. This evidence clearly indicates that soluble factors are involved in promoting such migration. Previous studies using migration assays have reported that bone-derived factors such as TGFβ1, Epidermal growth factor (EGF), Insulin-like growth factor (IGF-1) and osteonectin could be chemoattractants of CaP cells [30, 45, 46]. Besides growth factors and bone ECM proteins, chemokines and their receptors have also been implicated in organ-specific metastasis. For instance, the chemokine receptor, CXCR4 and its ligand, CXCL12 were both reported to be elevated in metastatic CaP compared to normal or benign prostate tissues of patients samples [11, 47].

Before cancer cells invade the bone, they arrest in the capillaries of the bone marrow (BM) via adhesion on the endothelium. Cells are more likely to attach on the trabecular vessel wall because the vascular beds which form sinusoids are large in diameter, and hence lower the blood flow rate. Subsequent attachment is enforced by the interaction of CaP cell receptors and BM endothelium. Membrane receptors eg selectins, integrins and cadherins and vascular cell adhesion molecules (CAMs) are responsible for docking and locking of the CaP cells on the BM endothelium [30, 48]. After extravasation of CaP cells into the bone site, a ‘fertile’ environment is required for establishment of micrometastases. The bone stroma, which is rich with growth factors (eg TGFβ1, IGF, BMP, EGF) and bone matrix
proteins (osteocalcin, osteopontin, bone sialoprotein), promotes cancer growth and adhesion to the ECM via integrins. Moreover, the ability of CaP cells to produce bone matrix proteins as they acquire an osteoblast-like phenotype (osteomimicry) improves homing capability, adhesion and survival in the bone environment [31, 45]. Taken together, the ‘soil’ which is the metastatic site is crucial for nourishing the planted ‘seed' (circulating CaP cells) for establishment of secondary tumors.

2.1.5 Osteoblastic lesion

CaP bone metastasis is found in about 70% of advanced CaP patients, presenting skeletal lesions that differ from other solid malignancies. In general, bone metastasis involves osteogenesis (mineralised bone matrix formation by osteoblasts) as well as osteolysis (osteoclast mediated degradation of mineralised bone matrix) and the balance between the two processes varies in different malignancies. For example, breast cancers lean towards osteolytic metastasis whereas CaP tends to demonstrate predominantly osteoblastic lesions caused by osteogenesis [37, 45, 49]. The activities of osteoblasts and osteoclasts are directly or indirectly regulated by growth factors, cytokines and proteolytic enzymes secreted by both CaP cells and bone cells (osteoblasts, osteoclasts). The role of essential growth factors and cytokines are listed in Table 2.2 and illustrated in Figure 2.3. Similarly, cell-cell and cell-matrix interactions also contribute to the “vicious cycle” of continuous elevation of bone formation/bone remodelling and cancer growth (Figure 2.3).
Table 2.2. Soluble factors involved in bone metastasis. Summarised from previous reviews [30, 31, 39, 45, 46].

<table>
<thead>
<tr>
<th>Factors</th>
<th>Secreted by</th>
<th>Role in bone metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>CaP cells, bone cells</td>
<td>Stimulates angiogenesis, promotes growth of OB and CaP cells</td>
</tr>
<tr>
<td>IGF</td>
<td>CaP cells, embedded in bone matrix</td>
<td>Promotes growth of OB, increases bone matrix apposition</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Embedded in bone matrix</td>
<td>Promotes growth of CaP cells and osteoblasts</td>
</tr>
<tr>
<td>FGF1</td>
<td>CaP cells</td>
<td>Promotes growth of osteoblasts</td>
</tr>
<tr>
<td>ET-1</td>
<td>CaP cells</td>
<td>Mitogen for osteoblast progenitors</td>
</tr>
<tr>
<td>BMP</td>
<td>CaP cells, osteoblasts</td>
<td>Activates osteoblasts, promote growth of CaP cells</td>
</tr>
<tr>
<td>RANK</td>
<td>Osteoblasts</td>
<td>Binds with RANKL to initiate differentiation of osteoclast progenitors to osteoclasts</td>
</tr>
<tr>
<td>RANKL</td>
<td>Osteoclast progenitors</td>
<td>Binds with its receptor RANK</td>
</tr>
<tr>
<td>OPG</td>
<td>CaP cells</td>
<td>Competes with RANKL for binding of RANK</td>
</tr>
<tr>
<td>IL</td>
<td>Bone cells, CaP cells</td>
<td>Mediates osteoclast formation</td>
</tr>
<tr>
<td>PSA</td>
<td>CaP cells</td>
<td>Degrades bone matrix and release active GFs</td>
</tr>
<tr>
<td>uPA</td>
<td>CaP cells</td>
<td>Degrades bone matrix and release active GFs</td>
</tr>
</tbody>
</table>
Figure 2.3. Osteoblastic bone metastasis mediated by CaP cells. In the bone environment, CaP cells secrete enzymes such as PSA, MMP and uPA that can degrade the bone matrix to create space for CaP cells to dock and grow. When the ECM is degraded, bound inactive growth factors like TGFβ and IGF-1 are released from the matrix. CaP cells also produce GFs that stimulate growth of osteoblasts eg TGFβ, IGF-1, Fibroblast growth factor (FGF), VEGF and Endothelin (ET-1), which enhance bone formation. Other soluble factors (e.g. Interleukin, IL, Parathyroid hormone-related protein, PTHrP, and Osteoprotegerin, OPG) produced by CaP cells also promote osteoclastogenesis by enhancing binding of receptor activated NF-kappaB (RANK) and receptor activated NF-kappaB ligand (RANKL), which then induces differentiation of osteoclast precursors to mature osteoclasts. This continuous series of events is also known as the "vicious cycle". As CaP cells invade the bone matrix, growth factors secreted by osteoblasts and embedded in the bone matrix stimulate CaP growth, which further induces osteoclastogenesis, matrix degradation and osteoblast growth. An increase in osteoblasts drives production of bone matrix and growth factors that further augments growth of CaP cells. Therefore, these events only exacerbate the condition. The net effect of osteogenesis and osteolysis cause an increase in bone formation.
2.1.6 Androgen and androgen receptor in CaP and CaP progression

Androgens are responsible for development of the male reproductive system including the prostate gland. Dihydrotestosterone (DHT) in particular is important during development of the prostate as early as 10 weeks of gestation. The androgen receptor (AR) only start to be expressed by prostatic epithelium in the early neonatal development during the time when prostate morphogenesis is near completion and initiation of secretory protein synthesis [50]. In the developed prostate, androgens activate AR signalling pathway that triggers other downstream activities related to enhanced cell survival and proliferation. Other downstream effects include upregulation of target genes such as PSA, also a biomarker for CaP at transcriptional and translational level [51]. During the development and progression of CaP, both androgens and the AR are involved in maintenance of CaP cells. Similar to normal prostatic epithelium, cancer cells too require androgens for their survival before they progress to become androgen independent CaP cells. Even at low androgen levels, the AR of androgen-independent CaP cells is still functional. In fact, the AR is more likely to bind to non-specific ligands and is more sensitive towards androgens [50, 52]. Thus, intervention in the AR signalling pathways by abrogating the AR activity is also a potential target for CaP therapy [53, 54]. In line with developing and testing new chemotherapeutic drugs, a physiological system is greatly needed for this purpose. Such systems will allow better interpretation of drug efficacy in vitro before proceeding to clinical trials.

2.2 3D models in cancer research

2.2.1 Transition from in vitro 2D cultures to 3D cultures

In the natural cell microenvironment, cells are mostly embedded and surrounded by the ECM and stroma. The ECM interacts with cells and relays mechanical and biological cues to cells which are then translated into physiological reaction. Hence, the biophysical and biochemical properties of the matrix are essential in dictating the behaviour of cells namely cell growth, differentiation, migration and signalling pathways. [55-60]. For example, biochemical entities such as ligands (e.g ECM proteins, GFs, cytokines and
hormones) present in the matrix influence cell adhesion, growth, differentiation and migration among other cellular responses. Similarly, the biophysical property of the matrix such as the stiffness, porosity and fibre architecture also induce cellular and molecular responses [61-65]. Two studies convincingly showed that by reducing the matrix stiffness, the malignant phenotype of human breast cancer cells can be reversed in vitro and tumorigenesis is significantly reduced in mice [66, 67]. This proves that while genetic aberrations may play a major role in carcinogenesis, the microenvironment clearly contributes to cancer development as well. Hence, to better understand the underlying cancer biology, there is a need to employ models that mimic the cells native microenvironment.

So far, the model that could best represent the microenvironmental factors (Figure 2.4) and physiological mechanisms are animal models. However, these models also have their limitations. Besides poor reproducibility of results that are attributed to heterogeneity of genetic makeup of the host, disease progression in humans may not be accurately represented in animal models. Moreover, animal hosts add complexity to a study by contributing multivariable factors that could interfere with a specific factor of interest. To avoid these problems, the system can be simplified to in vitro cultures, which allow better control over these conditions. The conventional in vitro model relies on two-dimensional (2D) cultures. This monolayer culture inevitably lacks the 3D cell-matrix interaction and is far from representing the microenvironment of native tissues. This alone creates diversion from the original cell phenotype (Table 2.3).

In order to bridge the gap between 2D and in vivo models, 3D in vitro models are being developed to capture some of the essential criteria of both 2D and in vivo models as listed in Table 2.4. In 3D models, cells can be embedded within matrices resembling the natural ECM, thus allowing cell-ECM interaction in a less complex and more well-defined condition. Particularly in cancer research, numerous studies have demonstrated that in vitro 3D tumors closely recapitulate in vivo growth
Figure 2.4. Microenvironmental factors that affect cell behaviour. These factors are taken into consideration when developing a 3D *in vitro* model to mimic *in vivo* microenvironment. Adapted from Yamada and Cukierman (2007) [62].

Table 2.3. Differences between cells in 2D culture and the native tissue. Summarised from previous reviews [10, 62, 68-70].

<table>
<thead>
<tr>
<th>Biological functions</th>
<th>Changes in 2D culture compared to native tissue</th>
<th>Regulatory mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape and polarity</td>
<td>Loss of epithelial cell polarity and changes in cell morphology</td>
<td>Cell surface receptor-ligand interactions (integrin-ECM, growth factor receptor- GF)</td>
</tr>
<tr>
<td>Growth</td>
<td>Usually faster</td>
<td>Adhesion and growth factors</td>
</tr>
<tr>
<td>Morphogenesis</td>
<td>Unable to form tissue-like structure ie sprouting, gland branching, lumen formation</td>
<td>Cell-ECM interactions, adhesion, GF</td>
</tr>
<tr>
<td>Motility</td>
<td>Altered. Migration is limited to one plane</td>
<td>Cell-ECM interactions, adhesion, biochemical gradient, growth factor</td>
</tr>
<tr>
<td>Gene expression</td>
<td>Altered</td>
<td>Biochemical and biophysical factors</td>
</tr>
<tr>
<td>Metabolic profile</td>
<td>Altered</td>
<td>Biochemical and biophysical factors</td>
</tr>
</tbody>
</table>

Table 2.4. Comparison between 2D cultures, 3D *in vitro* cultures and *in vivo* models. Summarised from previous reviews [10, 55, 62, 64, 69].

<table>
<thead>
<tr>
<th>Criteria</th>
<th>2D culture on plastic/glass surface</th>
<th>3D <em>in vitro</em> culture</th>
<th><em>In vivo</em> model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geometry of cell placement</td>
<td>Cells are cultured on top of substrata</td>
<td>Varies depending on the matrix or scaffold and devices available</td>
<td>Varies depending on the tissue eg circulating cells, cancer cells in ascites, endothelial cells, basal membrane, solid tumors</td>
</tr>
<tr>
<td>Stiffness of matrix</td>
<td>2-4 GPa</td>
<td>Modifiable from 100 Pa- MPa</td>
<td>100 Pa- MPa (non skeletal tissues, &gt;10 MPa (skeletal tissues)</td>
</tr>
</tbody>
</table>
characteristics and resistance against chemotherapeutic agents as seen in clinical settings [71-73].

From the above table, it is clear that the microenvironmental factors of 3D cultures more closely resemble the *in vivo* situation compared to 2D cultures. Therefore, cancer biologist and tissue engineers are already actively collaborating in an attempt to integrate the microenvironment complexity into *in vitro* models. This is greatly facilitated by the technology developed by the tissue engineering field.

### 2.2.2 Tissue engineering technology platforms for advancing cancer research

For the past years the transfer of the tissue engineering (TE) technology platform to the cancer research field has greatly encouraged the transition towards 3D cultures. Biomaterials such as natural or synthetic matrices/scaffolds (e.g. Poly(DL-lactic-co-glycolic) acid, PLGA, chitosan, alginate and collagen) traditionally used for engineering nerve, bone, cartilage tissues [74-78] have also been employed for culturing cancer cells [72] [79]. The bioinspired materials together with tissue culture techniques have enabled recreation of *in vitro* engineered tumors that are pathologically relevant to the human disease.

The application of 3D cultures in cancer research is numerous and can further expand our knowledge regarding roles of chemical cues, cell-cell interactions, cell-ECM interactions and mechanical signalling in tumor progression. In 3D cultures, ligands such as growth factors coupled to

---

<table>
<thead>
<tr>
<th>Nutrient/oxygen gradient</th>
<th>Nutrient and oxygen not limited</th>
<th>Depends on diffusion of molecules through matrix</th>
<th>Depends on vascular supply, dynamic spatial gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction with matrix</td>
<td>Only interact with attached surface</td>
<td>Interact with surrounding matrix</td>
<td>Interact with surrounding ECM</td>
</tr>
<tr>
<td>Interaction with stroma cells</td>
<td>Possible in co-culture, however limits the biochemical function analysis</td>
<td>Possible in co-culture. Allow analyses: -biochemical -migration -morphogenesis</td>
<td>Interact with stroma cells comprising endothelial, fibroblast, myoepithelial cells in a complex system. Difficult to study specific cell-cell paracrine interactions</td>
</tr>
</tbody>
</table>

*Pa= Pascal
polymeric drug delivery vehicle (eg. PEG) can be presented to cells in a controlled manner that allows studies on angiogenesis/migratory changes in response to these cues [80-82]. Scaffolds or matrices functionalised with ECM protein motifs for example, RGD (fibronectin) and YIGSR (laminin) on the other hand can be used to study cell-ECM interaction and changes in integrin signalling. In parallel to this interaction, by modifying the matrix stiffness the mechanical signalling is also effectively altered. To investigate the bidirectional intercellular interaction between cancer cells and the stromal microenvironment, multiple cell types such as endothelial cells, tumor associated fibroblasts, bone stromal cells and progenitor cells can be incorporated into 3D cultures. In line with the aim to recreate a physiological tumor model, it is desirable to develop tissue engineered tumor models that approximate the 3D structure, stromal environment and ECM milieu of in vivo conditions.

2.2.3 Utilization of 3D in vitro culture

As early as 1985, Miller et al. had already started using 3D cultures for studying drug sensitivity of mammary tumor cells in collagen gels [83]. Since then, other 3D models have been developed for cancer research but only for the past decade has utilization of these models been more forthcoming. There are various 3D culture systems used currently for culturing non tumor and tumor cells (Table 2.5). To date, spontaneous self-aggregation via liquid-overlay and rotating wall vessel methods are some of the common approaches used to form cell aggregates. These cell aggregates are usually spherical, compact in structure and also known as spheroids [3, 4, 84-88]. In addition to spontaneous cell aggregation, other methods such as embedding cells in matrices are also being explored to establish 3D models that can better represent the cell’s native microenvironment and at the same time address pertinent biological questions (Figure 2.5 and Table 2.5). For instance, 3D aggregates cultured in matrix may be more suited for studies related to most solid tumors e.g. CaP and breast cancer, which in their natural form are embedded within an ECM rich environment that form interactions with cells.
Matrices used for embedding cells not only provide a 3D physical support for cells but also create a microenvironment that forges cell-ECM interactions. Besides that, when cells are cultured within matrices, they develop into a multicellular mass that resembles early stage of tumorigenesis before a vascular network appears. To date, numerous matrices have been used for culturing cancer cells but only a selected few are shown to be compatible for inducing spheroid formation. These matrices are further discussed in the following section.

Table 2.5. Summary of 3D cell culture systems utilized for cancer research. Compiled from previous reviews [3-5].

<table>
<thead>
<tr>
<th>System</th>
<th>Description</th>
<th>Investigations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self aggregation</td>
<td>Spherical aggregate of cells in static (liquid-overlay) or stirred suspension culture (rotating wall vessel). Cells are prevented from attaching to the surface, hence promote aggregation.</td>
<td>Tumor cell biology, therapy resistance, cell-cell interactions, invasion, drug penetration, modeling, tumor markers, nutrient gradients, tumor cell metabolism. Drug transport and binding, therapy resistance, invasion.</td>
</tr>
<tr>
<td>Cellular multilayer</td>
<td>Layers of cells cultured on top of a porous membrane</td>
<td>Drug transport and binding, therapy resistance, invasion.</td>
</tr>
<tr>
<td>Matrix-embedded culture</td>
<td>Single cells or aggregates embedded in a porous extracellular matrix</td>
<td>Tumor cell biology, cell-cell interactions, cell migration and culture invasion.</td>
</tr>
<tr>
<td>Hollow-fiber bioreactor</td>
<td>Cells cultured within a network of perfused artificial capillaries</td>
<td>Tumor cell metabolism, therapy resistance.</td>
</tr>
<tr>
<td>Scaffold based</td>
<td>Cells attach to the scaffold/fibers. As the cells proliferate, they fill the pores to form 3D structures</td>
<td>Angiogenic capacity, cytotoxic drug response.</td>
</tr>
<tr>
<td>Microfluidic device</td>
<td>Cells assemble within a fluidic compartment. Size of aggregates and perfusion can be controlled.</td>
<td>Tumor cell biology, cell-cell interactions, drug toxicity.</td>
</tr>
<tr>
<td>Ex vivo culture</td>
<td>Pieces of tissue or tumor excised and cultured in vitro.</td>
<td>Therapy resistance, cell-cell interactions, tumor markers.</td>
</tr>
</tbody>
</table>

Figure 2.5. A schematic illustration of techniques used to induce multicellular aggregates/spheroids. (A) A liquid-overlay method where cells are seeded on agar coated surface to prevent cell attachment. (B) Spinner flask/rotating wall vessel cultures promote
cell aggregation by fluid turbulence. (C) Cells embedded within matrices form spheroids usually not by spontaneous aggregation but through cell division.

### 2.2.3.1 Matrix -embedded cultures

Cells embedded in matrix confer many characteristics of *in vivo* tumors particularly in terms of their morphology and architecture. Unlike the conventional suspended multicellular aggregates/spheroids, cells embedded within matrices make contact with neighbouring cells and the matrix that essentially influence the cell behaviour. Thus, matrix-embedded cultures are useful for studying normal and malignant cell differentiation, migration and tissue morphogenesis [6, 57, 89]. Matrices in the form of hydrogels consist of at least 95% water of the total mass and thereby very similar to the viscoelasticity of *in vivo* soft tissues. High water retention also allows maintenance of bioactivity of hydrophilic biomolecules [9, 90]. Hydrogels derived from natural ECM and synthetic ECM have different characteristics related to the compliancy, biochemical properties and versatility as listed in Table 2.6. As highlighted earlier, biological cues from the matrix play a crucial role in governing cell behaviour, hence, the matrix selection for 3D cultures is also of high importance and should be carefully considered. In the past, the first generation matrices consist of ECM derived from tissues such as basement membrane extract of mouse Engelberth Ewing sarcoma (Matrigel™) and collagen type I. Both matrices are gold standard natural ECMS for cancer cell cultures in 3D hydrogels. Matrigel™ is rich in ECM proteins (e.g. laminin, collagen IV, heparin sulphate proteoglycan), essential GFs (FGF, TGFβ, IGF-1, platelet-derived growth factor, PDGF, EGF) as well as other undefined components. Bissell and colleagues showed functional

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Natural ECM</th>
<th>Synthetic matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stiffness</strong></td>
<td>May or may not be modifiable. Stiffness of Matrigel™ cannot be altered while collagen gels can be chemically crosslinked to yield different matrix stiffness.</td>
<td>Easily modified.</td>
</tr>
<tr>
<td><strong>Concentration and type of</strong></td>
<td>Incorporation of other biomolecules is not feasible.</td>
<td>Can be readily tuned. The inert material itself</td>
</tr>
<tr>
<td>biochemical component</td>
<td>Matrigel™ contains mixture of ECM proteins (ie laminin, collagen type IV) and growth factors (FGF, Platelet-derived growth factor receptors, VEGF) that trigger multiple cellular responses.</td>
<td>does not induce cellular response.</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Polymerization mode</td>
<td>Chemical or physical crosslink. Gelation of Matrigel and collagen is very quick as temperature increases.</td>
<td>Chemical or physical crosslink. Gelation time is much longer allowing easier gel casting.</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Matrigel™ and collagen are highly viscous, hence homogenous cell distribution in the matrix is compromised.</td>
<td>Low viscosity allowing homogenous cell distribution.</td>
</tr>
<tr>
<td>Source</td>
<td>Natural sources e.g. animals and seaweeds.</td>
<td>Synthetically engineered from inert materials.</td>
</tr>
<tr>
<td>Batch to batch variation</td>
<td>High. Poorly defined content..</td>
<td>Low. Well controlled synthesis. Well defined content.</td>
</tr>
</tbody>
</table>

differentiation and polarization of normal mammary cells, forming lumens that secrete milk proteins in the Matrigel™ [92-95]. Matrigels can also induce polarization and acini morphogenesis in some ovarian, prostate and endometrial malignant or normal epithelial cells [96, 97] [98]. However, the complex mixture of ECM proteins and growth factors in Matrigel™, further triggers multiple cellular responses, which complicates the identification of specific inducing factors.

Collagen gels are biologically better defined than Matrigel and can be manipulated to a limited degree through changes in concentration, orientation of collagen fibrils, and biochemical modification [99, 100]. Of all 14 types of collagen, collagen type I is found most abundantly in mammalian connective tissue. The crosslinked collagen type I fibrils provide physical support to cells and at the same time play a biological role in interacting with cells through integrin receptors making it a promising biomimetic matrix [101]. Collagen type I can be extracted from rat tail or bovine skin and is used extensively for culturing breast, prostate, endometrial and lung cancer cells [102, 103] [104, 105]. Although the composition of collagen I gels are more defined unfortunately, numerous epithelial cell types fail to form polarized acini when cultured in this gel. On the contrary in Matrigel where basement membrane proteins are abundant, cell polarization can be induced [91]. These natural
matrices may resemble natural ECMs, however there are a few setbacks using them for cell culture.

Inconveniently, the biochemical and biophysical properties of Matrigel™ and collagen type I cannot be independently altered. For instance, altering the stiffness of collagen gels will also change the collagen ligand density. Matrigel™ stiffness on the other hand cannot be modified and remains in the range of 300-600 Pa (tested by our group) which restricts experimental parameters. Due to this reason and other disadvantages of natural hydrogels (Table 2.6), this has lead to the development of synthetic matrices that allow design of hydrogel properties to suit specific experimental purposes. Even though the ECM-like proteins are lacking in synthetic hydrogels, ligands with RGD motifs or equivalent peptides (eg. gelatin derivatives), MMP cleavage sites and growth factors can be conjugated into the hydrogel network in a defined concentration to yield a functional ECM [106-109]. The versatility of synthetic hydrogels has greatly facilitated addressing specific questions as demonstrated in a study by Loessner et al. (2010) [8]. They showed that the growth and morphology of the epithelial ovarian cancer spheroids are dependent on adhesion to the matrix via integrin-RGD interaction and the ability of the cells to degrade the matrix. They also used the 3D spheroid model for testing drug resistance and found that the ovarian cancer spheroids are more resistant to the anticancer drug, paclitaxel compared to monolayer cultures [8]. Clearly, the application of 3D cultures in the cancer field is numerous and can be modified to recapitulate the different stages of cancer progression.

2.2.3.2 3D in vitro bone metastasis model

In search of delineating cellular and molecular mechanisms of bone metastasis, numerous studies have been employed to identify contributing factors that trigger cancer progression. Due to the complexity of the bone metastasis process and the dynamics of bone remodelling, the disease is very often studied in animal models. A simpler model, the in vitro 2D based assay is also widely used for studying cancer-bone interactions. This usually involves either direct or indirect co-culture of cancer cells with osteoblasts/bone marrow stromal cells in 2D [110-114]. Besides studying
cell-cell interactions, cell-matrix interactions are also investigated in 2D cultures. For example, the decellularised bone matrix was used to investigate CaP cells and bone microenvironment interactions [115]. To narrow down specific factors of the bone matrix that influence cell behaviour, Ruppender et al. (2010) [116] had used a synthetic matrix that mimicked bone tissue rigidity to study the effect of the matrix stiffness on the expression of a pro-osteolytic marker. They have reported that the high matrix stiffness (3.3 MPa) induced expression of PTHrP and morphological changes of the breast cancer cells, MDA-MB-231, when compared to the softer matrix (0.45 kPa). These results can be correlated to the cellular response in a bone microenvironment where breast cancer is known to cause predominantly osteolytic lesions in bone metastasis sites [116]. These in vitro experimental setups that emulate certain characteristics of the bone microenvironment are able to provide valuable biological relevant data, however are lacking in the three dimensionality aspect. Due to the geometrical limitation of 2D cultures, interpretations of 2D based experiments also poorly represent cellular responses that involve migration and invasion.

While 2D cultures lack the essential 3D microenvironment factors of the in vivo condition, 3D cultures can compensate for this limitation. Thus, with the growing awareness of the importance of 3D culture systems in cancer research, a few attempts to create such models to answer biological questions pertaining to bone metastasis have been undertaken (Table 2.7). With the utilization of appropriate scaffolds and matrices, cells can be grown in 3D. Besides providing structural support to cells, 3D models also allow co-culturing of multiple cell types and examination of cancer cell migration, and various biochemical analyses. This is very well exemplified by a few previous studies involving cell-cell interactions. The importance of cellular interaction between cancer cells and the bone stromal cells are highlighted in direct co-culture studies where the cancer cells and osteoblasts were in contact with each other [117-121]. The result of this interaction was demonstrated in a study by Rhee et al. (2001) [117], where karyotypic changes to LNCaP cells were observed. They also found elevation of LNCaP cells growth after being co-cultured with bone stromal cells (2-3 weeks) when compared to parental cells (Figure. 2.6). This direct co-culture approach is also employed in other
in vitro models by culturing cells in microfluidic systems and bioreactors (Figure 2.7 and 2.10) [119-121]. While it is advantageous to forge intimate cell-cell contacts, the subsequent procedure to isolate the two cell types for further analyses (e.g. gene and protein expression) would be difficult and may still yield a mixed cell population. To further avoid mixture of cell populations, monocultures are also considered and can be adapted to test bone metastasis-related hypotheses.

Table 2.7. Current 3D in vitro models used for bone metastasis-related studies.
<table>
<thead>
<tr>
<th><strong>3D Models</strong></th>
<th><strong>Results</strong></th>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotating wall vessel (RWV) Direct co-culture of LNCaP cells and human bone stromal cells/osteosarcoma cell line, MG63 (Figure 2.6)</td>
<td>- Form aggregates/organoids after co-cultured with bone stromal cells, higher anchorage-dependent and independent LNCaP cell growth compared to parental cells - RWV derived cells have higher tumorigenicity and metastatic potential in castrated mice - CaP cells have less dependence on exogenous androgen and growth factors - Phenotypic and genotypic changes to MG63 cells, expression of ECM protein increase</td>
<td>- Promotes cell aggregation to form 3D structure - Forges direct interaction between CaP and bone stromal cells</td>
<td>- Difficult to separate two different cell types for further analysis - Lacking in similarities to the native bone microenvironment - 3D cell-matrix interaction is absent</td>
<td>[117, 118]</td>
</tr>
<tr>
<td>Microfluidic system Direct co-culture of PC3 cells and murine pre-osteoblast cells, MC3T3-E1 (Figure 2.7)</td>
<td>- Form spheroids only in co-culture - Slow growing in co-cultures as compared to 3D monocultures</td>
<td>- Culture condition is well controlled - Cells aggregate in microchannels of defined sizes to form spheroids - Cell distribution and spheroid size are uniform - Forges direct interaction between PC3 and MC3T3-E1 cells</td>
<td>- Difficult to separate two different cell types for further analysis - Lacking in similarities to the native bone microenvironment - 3D cell-matrix interaction is absent</td>
<td>[119]</td>
</tr>
<tr>
<td>Bilayer collagen gel PC3 cells in a layer of collagen gel co-cultured with human bone marrow endothelial cells, HBME-1, in another layer</td>
<td>- Increase in proliferation of PC3 in co-culture - Increase in migration of PC3 to HBME-1 layer - HBME-1 formed vessel-like structure (induces angiogenesis) in co-culture</td>
<td>- Allows separation of two cell types - Able to detect cell migration - Forges semi-direct interaction between PC3 and HBME-1 - Forges 3D cell-matrix interaction</td>
<td>- Collagen gel does not induce blood vessel-like structure for endothelial cells</td>
<td>[103]</td>
</tr>
<tr>
<td>Silk fibroin PC3 cells cultured in</td>
<td>- Increase in migration of PC3 towards BMP-2 coupled scaffolds</td>
<td>- Specific interaction with specific osteogenic GFs</td>
<td>- Absence of bi-directional CaP- bone cell interaction</td>
<td>[122]</td>
</tr>
<tr>
<td>Silk fibroin coupled with BMP-2 (Figure 2.8)</td>
<td>- Up-regulation of Col, Osteocalcin in PC3 - Forges 3D cell-matrix interaction - Extraction of cells for analysis is easy as only one cell type is involved</td>
<td>- Resemblance of scaffold stiffness to bone is questionable</td>
<td></td>
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<tr>
<td>Mineralised scaffold MDA-MB-231 breast cancer (BC) cells cultured in 3D porous PLG scaffold mineralised with HA (Figure 2.9)</td>
<td>- BC cells adhere and proliferate better on mineralised scaffolds - Conditioned media from BC cultured on mineralised scaffold promotes osteoclast differentiation and activity - Increase in IL-8 production</td>
<td>- Specific interaction with a component of bone matrix - Well characterised scaffolds with adjustable stiffness and HA deposition - Stiffness of scaffold 1.1MPa comparable to bone environment - Extraction of cells for analysis is easy as only one cell type is involved</td>
<td>- Absence of bi-directional BC-bone cell interaction [123]</td>
<td></td>
</tr>
<tr>
<td>Bioreactor Direct co-culture of breast cancer cell (BC) line, MDA-MB-231 and murine MC3T3-E1 cells (Figure 2.10)</td>
<td>- BC cells form colonies in immature osteoblast-like tissue (OT) - BC cells infiltrate mature OT (&gt;30 days) and align as “Indian filing” pattern, a characteristic of BC invasion - Metastasis suppressed MDA-MB-231 cells form smaller colonies - Degradation of OT when co-cultured with BC - Increase in IL-6 and decrease in OCN in media of co-culture</td>
<td>- Continuous culture without nutrient fluctuation - OT produces bone matrix proteins - Forges direct BC-OT and cell-matrix interactions - Able to detect cell migration and matrix remodelling</td>
<td>- Difficult to separate two different cell types for further analysis. Consequently, conditioned media is used which does not fully represent MDA-MB-231-MC3T3-E1 bi-directional interaction [120, 121]</td>
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Figure 2.6. CaP-stroma cultured in the rotating wall vessel. (A) A schematic of LNCaP cells cultured under simulated microgravity conditions with either microcarrier beads alone (RWV1), or with prostate (RWV2) or bone fibroblasts (RWV3) in a rotating wall vessel. (B) Cells derived from the RWV cultures were replated on tissue culture plates for assessment of growth profile. Growth profile shows an increase in cell proliferation of LNCaP cells from RWV1, RWV2 and RWV3 compared to parental LNCaP after being co-cultured with prostatic fibroblasts and bone fibroblasts. Adapted from Rhee et al. (2001) [117].
Figure 2.7. CaP-bone cultured in the microfluidic device. Schematic illustrations of the microfluidic device design (a–b) and spheroid formation process of DsRed transfected PC3 cells (PC3DsRed) and MC3T3-E1 co-culture (c). The device consists of two poly (dimethylsiloxane) PDMS microchannels separated by a semi-permeable polycarbonate membrane with 5 μm pores. The upper channel is a dead-end channel with 28 side-chambers to culture spheroids, and the lower channel has flow through capability for culture medium. Before seeding the cells, channels and membrane surfaces are rendered resistant to cell adhesion. The heterogeneous mixture of PC-3DsRed and support cells, MC3T3-E1 at 1:100 co-culture ratio were introduced into the upper channel as a confluent monolayer. The cells preferentially settled inside the side-chambers and self-aggregate to form PC-3DsRed/MC3T3-E1 co-culture spheroids within 1 day of culture. A section of confocal fluorescent image of PC-3DsRed co-culture spheroid illustrating the viability of PC3DsRed cells. Red = PC3DsRed cells, Green = Live cells (Calcein-AM stain), Yellow = Live PC-3DsRed cells. Adapted from Hsiao et al. (2009) [119].
In 3D monocultures without the presence of bone stromal cells, specific roles of bone components with regards to cancer progression can be independently defined. Some groups have opted for this approach by culturing cancer cells in scaffolds or matrices that emulate the organic and inorganic components of bone matrices. As demonstrated by Kwon et al. (2010) [122], BMP-2 (a growth factor that induces bone formation) coupled silk fibroin stimulates directional migration of PC3 CaP cells towards the functionalised silk fibroin (Figure 2.8) [122]. The inorganic component of bone matrix such as HA is also studied in a 3D bone metastasis model [123]. In this study, mineralised poly(lactide-coglycolide) (PLG) scaffold containing HA were cultured with breast cancer cells, MDA-MB-231 (Figure. 2.9). It shows that HA promotes adhesion and proliferation of MDA-MB-231 cells with increased osteoclastogenesis potential. Other bone matrix properties (e.g. mechanical property) can also be investigated in a monoculture system. It is also important to take into consideration the matrix stiffness when designing a bone metastasis model as the mechanical property also affects many aspects of cancer cell behaviour.

Since receptor-ligand interactions between cells and surrounding matrix are also implicated in development of bone metastasis, it is only apt that this feature is not compromised in a bone metastasis model. Some of the 3D direct co-culture studies described earlier (Figure 2.6 and 2.7) lack the cell-matrix interactions as cells are cultured in suspension (e.g. rotating wall vessel, RWV and microfluidic systems). Although these approaches promote formation of 3D structures, organoid/spheroids, this method for co-culturing cancer cells and bone stromal cells may not fully reflect the true condition of cancer cells in a bone microenvironment. The reason is because cells that are suspended in culture media are not surrounded by the matrix, hence lacking in 3D cell-matrix interactions. On the contrary, in bone metastasis, cancer cells are anchored to the ECM via cell surface proteins such as integrins and cadherins [39, 124-126]. Despite obvious improvements in the 3D bone metastasis model, many other issues still need to be addressed.
Figure 2.8. CaP cells cultured on silk fibroin coupled with BMP-2. (A) The design of a 3D model system \textit{in vitro} and 3D porous aqueous-based scaffold (pore size, 1000–1180 μm). Two scaffolds were attached, fixed using a needle and rubber mat to stabilize them in medium. (B) 3D porous aqueous-based silk scaffold cut to size 8 × 3 mm (diameter × height). (C) Projections of composite single and two photon-excited fluorescence image stacks of PC3 on silk scaffold taken over approximately 155 μm. PC3 cells were labelled with diakylcarbocyanine (DiI) (red). (D) DNA quantification of the top scaffolds showed that the migration of PC3 cells from the bottom scaffold to the top scaffolds which were coupled with BMP-2 is greater than the non coupled silk scaffold. Silk scaffolds which are used for engineering of bone tissue are also used in cancer studies as demonstrated in \textit{in vivo} models. (E) Histological examination of human bone marrow stromal cell (BMSC) seeded scaffold explants. Silk scaffolds were seeded with human BMSCs and osteogenically induced before being implanted subcutaneously into NOD/SCID mice. Inoculation with BC cells SUM1315 via mammary fat pad was performed 4 weeks post scaffold implantation. Mice were sacrificed at 3 months after SUM1315 injection for assessment of metastatic spread. Scaffold cross-sections labelled with cytokeratin 5/6 (brown) confirms the presence of invading SUM1315 cells at the scaffold perimeter. Scale bars, B= 2 mm, C= 150 μm and E= 100 μm. Adapted from Kwon \textit{et al.} (2010) and Moreau \textit{et al.} (2007) [122, 127].
Figure 2.9. Breast cancer cells cultured on HA scaffolds. (A) 3D poly (lactide-coglycolide) (PLG) scaffolds used for culturing breast cancer (BC) cells, MDA-MB-231. Physicochemical characterization of scaffolds shows that incorporation of HA did not alter the scaffold microarchitecture relative to non-mineralized control scaffolds as indicated by visualization via brightfield microscopy. (B) Quantification of DNA indicates enhanced proliferation of MDA-MB231 cells within mineralized scaffolds as compared to non-mineralized scaffolds. (C) Live and dead staining with calcein (green) and propidium iodide (red), respectively, shows increased cell number and tissue formation into pores of mineralized scaffolds relative to control scaffolds. Scale bars, A= 2 mm and C=100 μm. Adapted from Pathi et al. (2010) [123].

In general, the 3D bone metastasis models are still very diverse and constantly evolving. More advanced culture systems are now being employed such as using bioreactors or microfluidic channels, where continuous media exchange is performed (Figure 2.6, 2.7 and Figure 2.10). Importantly, a defined and constant nutritional milieu in the culture media renders steady state gene expression. This also improves reproducibility of experiments and functional comparisons since cell response is affected by fluctuation of solubles [128]. However, these systems require instruments and devices that are costly and complex to set up. Besides variations in cell culture systems used for bone metastasis models, the species of which bone
stromal cells were isolated from also differ. Some studies use cells of either mouse origin (e.g. MC3T3-E1) or of human origin. Not only has this contributed to the inconsistency between independent studies but also possibly lead to misinterpretation of results regarding human disease.

As shown by Dhujarti et al. (2008) [120] and Krishnan et al. (2010) [121], murine derived osteoblastic tissue generated from the bioreactor show bone tissue like characteristics by expressing bone matrix proteins (Figure 2.10). They also reported that MDA-MB-231 cells form colonies and are able to penetrate the murine osteoblast tissue (OT) when co-cultured in a bioreactor [120, 121]. Since their study utilizes a mixed species cell source, a
murine derived OT and human breast cancer cell line, this means that data has to be interpreted with caution. In order to improve clinical values of *in vitro* studies, besides mimicking the non cellular entity of the bone microenvironment, a matching species to human cellular component and bone tissues is also vital. As shown by Moreau *et al.*, when tissue engineered human bone (TEB) derived from human bone marrow stromal cells were implanted in mice, circulating breast cancer cells are able to home to the TEB site (Figure 2.8E). Therefore, using an all human model would better relate the outcome of the human cancer disease.

Engineered tissue constructs such as the bone construct that have been tested in animal models also inspire utilization of appropriate constructs for *in vitro* cancer studies. Parallel to *in vitro* studies, *in vivo* bone metastasis models are also continuously being modified to improve recognition of human cancer cells to metastatic sites. One example is using human bone fragments implanted subcutaneously into mice as a metastatic target for CaP cells [129, 130]. Due to variation in human bone, that may result in inconsistency of metastatic spread, engineered bone-like tissue provide an attractive substitute for native human bone. With the advances in TE, easily fabricated tissue engineered bone constructs are available for use in not just animal models but also for 3D *in vitro* models [120, 121, 127]. This robust bone-like tissue is shown to interact with cancer cells and vice versa in an *in vivo*-like manner [131]. The Hutmacher group has developed a human tissue engineered bone construct (hTEBC) that has been successfully used as scaffolds for cranial defect therapy [11]. In addition to regenerative medicine applications, the reproducible and robust construct is also being used for this study in line with the aim to develop a 3D *in vitro* bone metastasis model.
2.3 Summary

One of the many challenges in cancer research is applying relevant models for testing hypotheses. This has fuelled the development of in vitro models, more recently the 3D culture systems that have been proven to better represent the in vivo condition. Hence, the collaboration between tissue engineers and cancer biologist is greatly encouraged to fulfil this unanimous vision. In the context of CaP, only a handful of CaP studies are conducted in 3D models. In addition, monoculture studies still rely heavily on naturally derived matrices, which limit the application of this model. 3D models to study other aspect of CaP, such as bone metastasis is still lacking as most 3D in vitro bone metastasis models, are aimed to exemplifying breast cancer-bone interactions. Therefore this PhD project is designed to provide means for understanding CaP biology using a well characterized 3D in vitro system.

Hypothesis

Tissue engineering principles can be employed to develop 3D in vitro model systems which allow a more physiological study of CaP cells and osteoblast interactions than 2D cultures.

Aims

1. Establishment of a 3D model for culturing CaP cells by identifying optimal parameters for culturing CaP cells in PEG based hydrogels.
2. Characterization and comparison of the phenotype and gene expression of CaP cells in 2D and 3D cultures.
3. Establishment of a 3D co-culture model for studying CaP cells and human osteoblast interaction in a direct or indirect co-culture approach.
4. Application of the 3D co-culture to study the effects of synthetic androgen R1881 treatment on gene and protein expression.
Chapter 3: Phenotypic characterization of prostate cancer LNCaP cells cultured within a PEG-based synthetic and biomimetic matrix
Chapter 3: Phenotypic characterization of prostate cancer LNCaP cells cultured within a PEG-based synthetic and biomimetic matrix

Abstract

Biophysical and biochemical properties of the microenvironment regulate cellular responses such as growth, differentiation, morphogenesis and migration in normal and cancer cells. Since 2D cultures lack the essential characteristics of the native cellular microenvironment, 3D cultures have been developed to recapitulate the properties of natural ECM. To date 3D culture systems rely mostly on natural extracellular (ECM) hydrogels that are characterised by limited control over matrix stiffness and ligand density. In contrast, synthetic hydrogels offer the opportunity to independently tune these parameters, thereby allowing us to systematically investigate their influence on cell growth. In this study, we implemented polyethylene glycol (PEG)-based hydrogels functionalized with RGD motifs and matrix metalloproteinase (MMP) cleavage sites for LNCaP prostate cancer cell growth. Firstly, physical characterization (stiffness and diffusive behaviour) of the hydrogels was performed, and further compared in terms of LNCaP cell growth. The PEG content was found to modulate the growth kinetics of LNCaP cells, in that the lowest PEG concentrations (1.5%) induced the maximal cell growth. Next, we compared the morphology of LNCaP cells grown in 2D and 3D cultures. We observed that cells undergo morphogenic changes forming tumour-like structures in 3D cultures, with a hypoxic and apoptotic core in their centre. Notably, the fold change in gene expression levels of androgen responsive genes upon treatment with the synthetic androgen analogue R1881 in 3D cultures greatly differed from 2D cultures. Moreover, androgen receptor (AR) localised differently in 2D and 3D cultures. Such discrepancies suggest differences in regulation of androgen-related genes and AR activity. We suggest that the presented 3D culture system represents a powerful tool for not only to recapitulate initial stages of prostate cancer but also for high throughput drug testing.
3.1 Introduction

Prostate cancer (CaP) is one of the most prevalent malignant diseases among men in western countries. Whereas the 5-year survival rate for men diagnosed with localized CaP approaches 100%, the prognosis worsens rapidly upon CaP progression [132-134]. Despite much advancement in detection methods and treatments, CaP still remains a major cause of cancer death in men. Therefore, it is important to gain a greater understanding of the progression from localized to advanced CaP using relevant physiological systems.

Like many other cancers, CaP cells have been extensively studied in 2D cultures, through which a significant basic understanding of cancer biology has been unveiled. However, in native tissues cells are in a 3D ECM that provides not only architectural support, but also chemical and mechanical cues to cells that modulate cell signaling [135, 136]. In particular, over the last few years the importance of the mechanical properties of the tumor environment has been increasingly acknowledged. Herein, it is demonstrated that the stiffness of the microenvironment can modulate cancer cell growth, signaling and even facilitate tumor progression [56, 59, 63, 67, 137]. Considering the artificial geometric constraints and immensely high substrata stiffness imposed on cells on 2D tissue culture plastic, it is not surprising that 2D cultures fail to recapitulate fundamental aspects of tumor growth, such as cell polarity, morphology, migration and proteolysis mechanisms [58, 62]. Therefore, 3D culture systems have been increasingly applied to better mirror the in vivo microenvironment of tumors. In recent years, numerous studies have demonstrated that studying tumors in 3D better reproduces in vivo growth characteristics and resistance against chemotherapeutic agents than a 2D approach [71-73]. The most commonly used 3D matrix models are reconstituted basement membrane extract, Matrigel™ [72, 97, 138] and polymerized collagen type I matrices [10, 66, 104, 139]. Although these naturally derived matrices have ECM-like biological properties, their inherent characteristics limit the flexibility of adjusting matrix stiffness without simultaneously affecting other parameters such as ligand density. Matrigel™, containing a complex mixture of ECM
proteins and growth factors, further triggers multiple cellular responses, which complicates the identification of specific inducing factors. Furthermore, Matrigel™ shows batch-to-batch variations, which decreases the reproducibility of experiments. Considering the complexity of cellular interactions with naturally derived matrices, there is a growing interests in the use of more manageable systems that allow studying more systematically the impact of certain components or properties of the tumor microenvironment on tumor cells. Therefore, emerging approaches in biomaterial science have focused on the development of synthetic matrices such as hyaluronon-derived or alginate matrices for culturing cancer cells [107, 140, 141]. Another example is, PEG-based hydrogels that are inert themselves in terms of triggering cell signaling pathways, but can be equipped with biological functionalities, e.g. RGD motifs, in a controlled fashion [142]. Advantageously, the stiffness of such hydrogels can be precisely tuned independent of the ligand density.

We and others have used in the past a synthetic hydrogel PEG-based hydrogel, in which RGD motifs and peptides with an MMP cleavage sequence are incorporated at a defined density [8, 58, 109]. The RGD motif provides fibronectin-integrin binding sites for cells and the MMP sequence permits cells to degrade the matrix via proteolytic activity, which creates space for cell expansion and migration. This reflects events occuring in the in vivo situation [8, 57, 108, 142-144]. Our previous work has focused on interactions of ovarian cancer cells within these PEG hydrogels and we showed that this 3D culture system is highly reproducible, and suitable for studying cell morphology, proliferation and drug resistance more accurately than in conventional 2D cultures [8].

To this end, a thorough phenotypic characterization of CaP cells cultured within this synthetic ECM has not been reported. In this study, we aim to establish a 3D culture system that reflects the early stage of CaP development by growing LNCaP cells in the above mentioned PEG-based hydrogels. Firstly, we examined the effect of the hydrogel’s stiffness on cell growth to determine the optimal hydrogel composition for further experiments. Thereafter, morphology, gene expression and protein synthesis of LNCaP cells grown in 3D hydrogels was compared to conventional 2D
cultures. We also used this 3D culture system to study the effects of androgen mediated AR signaling activation by using the synthetic agonist, R1881, which has been previously studied extensively in 2D cultures [145, 146]. Our findings presented here provide insights into the role of the microenvironment in modulating cell behavior and highlights the discrepancies between 2D and 3D cultures. This model is a stepping stone for future development of 3D culture systems that will be essential for CaP biology studies in replicating the in vivo tumor microenvironment.
3.2 Materials and methods

Materials
The synthetic androgen, R1881 was purchased from DuPont. For immunostaining and Western blotting, primary antibodies against E-cadherin (Invitrogen), Pimonidazole (Hypoxyprobe), Caspase-8 (Abcam), androgen receptor C19 (AR, Santa Cruz), Cytokeratin 8 M20 (CK8, Abcam), and Prostate specific antigen (PSA, Dako cytomation) were used. Fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA) was kindly provided by Dr. Jonathan Harris.

Cell culture
The LNCaP prostate cancer cell line (American Tissue Culture Collection, Rockville, MD) was cultured in RPMI (Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Invitrogen). LNCaP cells were obtained from passage 18 and were subcultured 2-8 times before experimentation. For all 2D experiments, 1x10^4 cells/cm^2 were seeded on tissue culture plastic or glass cover slips.

Preparation of PEG-based synthetic hydrogels
PEG-based hydrogels were prepared as previously described by Ehbar et al. (2007) [108, 109, 142]. In summary, PEG-Gln/PEG-MMP-Lys precursor stock solution (5% w/v) was diluted to the desired PEG concentration in Tris-Buffer (50mM, pH 7.6) containing 50μM RGD (Arginine-Glycine-Aspartic acid) conjugate, and 50mM calcium chloride. Following the addition of 10U/mL thrombin-activated factor XIII, the LNCaP cell suspension was added into the reaction mixture to yield a final cell density of 3.5x10^5 cells/mL (Figure 3.1A), and hydrogel discs were formed by sandwiching 20μL drops of the reaction mixture between two sterile glass slides (separated by 1.5mm spacers) pre-coated with SigmaCote (Sigma). Hydrogels were allowed to polymerize at 37°C for 20-40min before being transferred to 24 well plates filled with growth medium. Medium was changed every 4 days.
Mechanical testing of hydrogels

To measure the global stiffness of cell-free hydrogels of 1.5, 2.0 and 2.5% PEG content, unconfined compression tests were conducted using an Instron microtester (Instron). Measurements were performed at 30% strain with displacement of 0.45 mm/min, using a 5 N cell load. During compression, hydrogels were maintained at 37°C in a moist condition. The elastic (Young’s) modulus was extracted from the linear region of the stress-strain curve (9-15%) according to the equation \( E = \frac{F}{A \cdot L / L_0} \). \( F \) = force applied to sample, \( A \) = cross section area which the force is applied, \( L \) = the amount of change in hydrogel thickness, \( L_0 \) = the original thickness of the hydrogel.

Local stiffness of the hydrogels was examined by atomic force microscopy (AFM) indentation measurements using a NanoWizard II from JPK instruments (Berlin, Germany). Pyramidal-shaped cantilevers (MLCT, Veeco) were calibrated in situ using built-in procedures of the AFM software. Coverslips with hydrogel discs were mounted into the temperature-controlled chamber of the instrument (Petridishheater, JPK instruments). The measurements were conducted at 37°C in PBS. To measure the elastic (Young’s) modulus, \( E \) of the PEG hydrogels, the cantilever was approached at a speed of 2 \( \mu \)m/sec onto the hydrogel until a contact force of 0.8 nN was reached. From the recorded force versus tip-sample-separation curves (Figure 3.1C, left), the elastic modulus was extracted using procedures implemented into the JPK IP software. This procedure applied a Hertzian fit assuming a pyramidal indenter with half-angle-to-face of 17.5°, and a Poisson’s ratio of 0.5. For each gel, the elastic modulus was measured on at least 50 different spots on the gel. At least four gels from independent preparations were analysed for each PEG concentration.

Diffusion measurements

Cell-free hydrogel reaction mixtures of different PEG contents were pipetted into a pasteur pipette of approximately 2 mm diameter and allowed to polymerize at room temperature for 20 min. Then, the food dye, E133 (792 Da) (1:1000 dilution) or FITC-BSA (66 kDa) (1 mg/mL) was injected into the pasteur pipette to come in contact with the hydrogel before incubation at 37°C. After 110 min (E133) and 50 min (FITC- BSA), the hydrogel-tracer
solution intersections were imaged using a Nikon Eclipse microscope equipped with a Nikon Digital camera DXM1200C (Coherent Scientific). From the grey scale images, an intensity profile was plotted over the region from the gel-dye solution interface to the dye-absent position using image J. The intensity profile was fitted to the following equation \( f(x) = a \cdot \text{erfc} \left( \frac{x}{\sqrt{4Dt}} \right) \) using IGOR Pro (wavemetrics).

\( D = \) diffusion coefficient, \( a = \) intensity of the tracer, \( x = \) distance of measured tracer from \( x_0 \) and \( t = \) duration of hydrogel exposure to the tracer.

**R1881 treatment for 2D and 3D cultures**

Both 2D and 3D cultures were maintained in androgen-deprived media (RPMI media containing 5% charcoal stripped serum) for 48 hr prior to androgen treatment. Androgen deprivation was initiated when cultures reached 70-80% confluency for 2D cultures and on day 24 for 3D cultures. Media were then changed to androgen-deprived media containing 1 nM R1881. The cultures were treated with R1881 for 48h before imaging or protein and RNA extraction. 2D and 3D cultures incubated for 96 hr in androgen-deprived media in the absence of R1881 were used as controls.

**Proliferation assay for 2D and 3D cultures**

For 2D cultures, cells were plated in 24 well plates and harvested daily from day 1 to day 7 by adding 200 \( \mu \)L of Proteinase K (0.5 mg/mL). The cell suspension was incubated at 56 °C for 12 hr and stored at -80 °C before a Pico Green (Invitrogen) proliferation assay was performed according to manufacturer’s instructions. For 3D cultures, hydrogels of different PEG concentrations were harvested at day 1, 7, 14, 21 and 28 and also subjected to digestion with 300 \( \mu \)L of Proteinase K for 16 hr prior to a Pico Green assay. The digested cell suspension mixture was diluted with phosphate buffered saline/ethylenediaminetetraacetic acid (PBS/EDTA) buffer to allow readout within the range of the standard curve between 2 \( \mu \)g/mL and 10 ng/mL DNA. All samples were assayed in triplicate from three biological samples.
Live-dead staining
At day 24 of 3D cultures, hydrogels with cells were washed with PBS and incubated in 2 μg/mL fluorescein diacetate (FDA, stains for live cells) solution diluted in PBS for 30 min at 37 °C. Cells were then incubated for another 5 min in 20 μg/mL propidium iodide (PI, stains for dead cells) solution before washing thoroughly with PBS. Hydrogels were immediately analysed using a Leica SP5 confocal microscope (Leica). Z stacks over a range of 150 μm were imaged and 3D projections were created using the Leica Image Processing software.

Phalloidin-DAPI staining and spheroid size and shape Characterization
Cells were fixed in 4% formaldehyde/PBS, permeabilised in 0.2% triton X and stained with 0.8U/ml rhodamine conjugated Phalloidin (Invitrogen) and 2 μg/ml 4’,6-diamidino-2-phenylindole (DAPI) [Invitrogen] for 40min as described [131]. Fluorescent confocal images stacks over a range of 100-200 μm were captured with Leica SP5 laser scanning confocal microscopy (CLSM). From the 3D projections, the size and shape factor of the spheroid were measured using Image J software.

Immunofluorescent staining
LNCaP cells were either cultured in normal growth media or treated with R1881 as described previously. For 2D cultures, LNCaP cells were cultured on glass cover slips. 2D and 3D cultures were fixed with 4% paraformaldehyde/PBS for 30 min. Hydrogels from 3D cultures were either stored in 4 °C or processed for cryosection. Hydrogels for cryosection were prepared by immersing them in OCT Tissue Teck/PBS (1:1) solution for 45 min followed by another 45 min in OCT Tissue Tek. Then, the hydrogels were loaded into cryomoulds, fully immersed in OCT Tissue Tek and frozen with liquid nitrogen. Hydrogels were stored at -80 °C until ready for sectioning. 7 μm thick sections were permeabilised with 0.2 % Triton-X for 10min and blocked for 1 hr with 1% BSA solution before incubation in primary and fluorescently conjugated secondary antibodies. Samples were
counterstained with Phalloidin and DAPI for 40 min. Images were captured using Leica SP5 CLSM (Leica).

**Immunohistochemistry and Haematoxylin & eosin (H&E) staining**

Frozen sections were processed as the immunofluorescent staining up to the secondary antibody incubation. Blocking endogenous peroxidise was performed before incubating the slides with horseradish peroxidase (HRP) conjugated secondary antibody. Immunoreactivity was detected with diaminobenzidine (DAB) solution. To counterstain the slides with H&E staining, they were then incubated with Mayer’s Haematoxylin for 1 min at room temperature and washed with water until slides were clear of excessive stain. Then slides were dehydrated in 70%, 90% and finally 100% for 3 minutes each. Following that, the sections were stained with eosin for 1 minute before being washed with 100% ethanol to obtain a satisfactory cytoplasmic stain. Slides were mounted and sections were imaged with Axio Zeiss microscope.

**Cell lysate preparation and Western blotting assays**

2D and 3D cultures of treated and non treated groups were harvested for lysate preparation in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA and 25 mM NaF) containing protease inhibitor cocktail (Roche). For 3D cultures, hydrogels were dispersed using a pipette to release the embedded spheroids. 20 μg of protein was loaded onto a 10% sodium dodecyl sulphate (SDS) polyacrylamide gel and separated by electrophoresis for 2 h at 120 V. Proteins were transferred to a nitrocellulose membrane by wet transfer for 1h at 100V. After primary and secondary horseradish-peroxidase conjugated secondary antibody incubation, chemiluminescent Pierce ECL Western Blotting Substrate (Thermo Scientific) was added and membranes were exposed on X-ray films.

**RNA isolation and quantitative Real-Time PCR (qRT-PCR)**

RNA extraction was performed with Trizol (Invitrogen) according to the manufacturer’s instruction. For 3D cultures, three hydrogels were pooled before adding Trizol reagent. RNA concentrations were quantified using a
Nanodrop-1000 (ND-1000). Samples with a 260/280 ratio higher than 1.7 were used for subsequent procedures. The samples were then treated with DNAsse Amp grade I and reverse-transcribed using the cDNA synthesis for qPCR kit (Invitrogen). QRT-PCR was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems) and data analysed with SDS2.3 software as described previously [131]. The sequences of all primers are as follows: GAPDH, 5'-GCAAAATTCCATGGCACCCTG-3' and 5'-TCGCCCACTTTGATTGGG-3', PSA, 5'-AGTGCGGAAGCATTTCACCAAC-3' and 5'-CCAGCAAGATCAGCTTTGTTTG-3', AR, 5'-CTGGCACGACAACCAACCCAG-3' and 5'-CAGATCGGGGCCAGATAGTA-3', and CK8, 5'-CTGGGATGAGAAACACGATTTC-3' and 5'-GTAGCTGAGGCCGGGGCTTGT-3'.

Microarray gene expression profiling

Triplicates of each condition were prepared for microarray profiling which was performed on a custom Agilent 4x180k oligo array. This microarray incorporates Agilent human gene expression protein-coding probes as well as non-coding probes; with the probes targeting exonic regions, 3'UTRs, 5'UTRs, as well as intronic and intergenic regions. RNA was isolated with Trizol, followed by clean-up using a RNeasy Mini Kit (Qiagen) and DNase on column treatment according to the manufacturer’s protocol. RNA samples were analysed by a Bioanalyzer (Agilent) to ensure the RNA was of high quality. 200ng of RNA from each group was amplified and labelled according to the protocol for One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling Kit, Agilent). The input RNA was reversed transcribed into cDNA, using an oligo-dT-promoter primer which introduces a T7 promoter region. The subsequent in vitro transcription uses a T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3-labeled CTP. cDNA synthesis and in vitro transcription were performed at 40 °C for 2 hr, respectively. The labelled cRNA was purified with
Qiagen’s RNEasy mini spin columns and quantified using a Nanodrop-1000. 1650 ng cRNA from each sample were loaded onto the array chambers and allowed to hybridize at 65 °C for 17 hr. The arrays were scanned with the Agilent Microarray Scanner G2565CA.

**Microarray data analysis**
The microarray data were processed with Agilent Feature Extraction Software (v10.7) and normalized using the quantile normalization method found in Linear Models for Microarray Data (LIMMA), an R programming language package. The gene expression levels are presented as log_{10} and were compared between two groups with a t-test. Genes that were significantly different between two groups were identified with a p value of <= 0.05, and an average fold change of >= 1.5. The androgen-responsive gene filter was generated from microarray data of DHT- and R1881-treated LNCaP 2D cell cultures and consists of 4702 genes commonly regulated by both androgens (M Lehman and CC Nelson, unpublished results). The microarray data from the 2D and 3D cultures were processed with the androgen-responsive gene filter to compare their regulation in response to treatment (R1881 versus ethanol) and culture conditions (2D versus 3D). The filtered gene lists were examined by Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc.) for functional annotation and gene network analysis.

**Statistical analysis**
Univariate ANOVA post hoc LSD tests were used to determine the statistical significance of data between more than two conditions for hydrogel stiffness, diffusivity, spheroid size and shape factor measurements, qRT-PCR and Western blot.
3.3 Results

**Mechanical and diffusive properties of PEG-based hydrogels are dependent on the PEG content.**

To mimic the early stage of CaP tumor formation in 3D, we set out to culture LNCaP cells in synthetic PEG hydrogels. The PEG precursors conjugated with MMP cleavage sites and RGD motifs were incorporated into the hydrogel network to confer essential features of the natural ECM (Figure 3.1A). Since mechanical properties of the hydrogel are known to influence growth, morphology, and invasive phenotype of cancer cells [56, 60, 147], we first aimed to optimise the hydrogel’s stiffness for cell culture. A variety of cell-free PEG hydrogel stiffness was prepared by adjusting the PEG content to 1.5, 2, 2.5%(w/v). The stiffness of the hydrogel was determined by unconfined compression tests conducted using a microtester (Figure 3.1B) and AFM indentation measurements (Figure 3.1C). While the microtester probes the global elastic properties of the PEG hydrogels, the AFM indentation measurements yield information about their local stiffness distribution (Figure 3.1C). Both methods reveal a linear increase in elastic moduli with augmentation of PEG content, ranging between approximately 0.8 kPa to 10 kPa for the 1.5% and 2.5% PEG hydrogels, respectively. AFM measurements present a fairly homogenous distribution of E moduli over the probed hydrogels (Figure 3.1C, middle). The local stiffness variation however is still within an order of magnitude of the average stiffness. When the 2.0% hydrogels (with cells) were tested with the microtester after more than 4 weeks of culture, we found no drastic changes in the matrix stiffness (2.5-4.2 kPa) which is consistent with the variation in the tested 2% hydrogels (Supplementary Figure 3.1). This proves that the PEG hydrogel is highly robust.
Figure 3.1. Characterization of PEG-based hydrogels (A) A schematic showing the preparation of PEG-based hydrogels. LNCaP cells were mixed with PEG precursors containing MMP cleavage sites and RGD peptides prior to FXIII-catalysed polymerization. (B) Global stiffness of cell-free hydrogels with different PEG content (w/v) was measured using a microtester. (Middle) A representative stress-strain curve recorded for a 2% PEG gel. The Young’s modulus, $E$, was calculated from the slope of the line fitted to the stress-strain curve at 9-12% strain. (Right) Scatter plot showing the Young’s moduli measured for
Changes in PEG content are likely to alter the pore size of the PEG hydrogels [148], which may significantly affect the transfer of small molecules, such as the synthetic androgen R1881, and also growth factors present in the medium in our experiments. To estimate the diffusion of R1881, we measured the diffusion coefficient D of the food dye E133 (792 Da), that is of higher molecular weight than R1881 (284 Da). We found that the median values of diffusion decreases with increasing PEG content (Figure 3.1D) from 2.99 ± 0.06 x10^{-6} cm²/s for 1.5% PEG hydrogels to 1.9 ± 0.13 x10^{-6} cm²/s for 2.5% PEG hydrogels. To ensure that essential growth factors such as FGF, VEGF and PDGF (20-50 kDa) can also penetrate the hydrogel, the diffusion of FITC-BSA (66 kDa) having a higher molecular weight than these growth factors, was then investigated. The diffusion coefficient of FITC-BSA is much lower compared to E133, but decreases analogously with increasing PEG content (Figure 3.1D). D values for 1.5-2.5% PEG hydrogels (1.5-3.0x10^{-7} cm²/s) are within the same order of magnitude (6x10^{-7}cm²/s) as reported by Ramanujan and Erikson measuring diffusion of BSA in the softer collagen gels [149, 150]. When PEG hydrogels were incubated with FITC-BSA for 24 hr, we observed a widespread penetration of the FITC-BSA (Supplementary Figure 3.2). From these findings we may conclude that although the transfer of R1881 and growth factors are hindered by the increasing PEG content, the pore size of all gels is sufficiently large for soluble factors from culture medium to reach the cells.
**Higher proliferation of LNCaP cells within hydrogels of lower elastic modulus**

To evaluate the effect of matrix rigidity on cell proliferation, we next performed proliferation assays on LNCaP cells cultured in 1.5, 2.0 and 2.5% hydrogels over 28 days (Figure 3.2A). Monolayer cultures (doubling time, Td = 27 hr) grow exponentially up to day 5 and started to plateau thereafter. On the contrary, cells proliferate more slowly in 1.5% (Td=54 hr) and 2% (Td=58.5 hr) PEG hydrogels. Exponential growth is only achieved after day 7 in 3D cultures. Although growth in 3D is less than 2D cultures at day 7, the final fold increase in 3D cultures is at least 4 times more than 2D cultures. Surprisingly, no growth is detected in 2.5% hydrogels as confirmed by the live-dead staining (Figure 3.2A, lower panel) that reveals presence of predominantly non viable cells in the 2.5% hydrogel at day 24. Bright field images show that cells are able to form colonies in the 1.5 and 2.0 % hydrogels while non viable cells in 2.5 % hydrogels remain single cells as they were first seeded. Based on this proliferation study and reported stiffness of normal and cancerous prostate tissue (17 kPa and 24 kPa, respectively) by others, we have selected 2.0% PEG hydrogels for conducting subsequent experiments. Although the stiffness of the hydrogel is lower than the reported prostate tissues, it is still in agreement with the stiffness of soft tissues range (1-10 kPa) and breast glandular tissue (4 kPa) [55]. In keeping with mimicking in vivo-like matrix stiffness that allow cell growth, this condition was implemented to emulate the early phase of tumor development when the surrounding stroma is softer than the advanced stage of CaP [151, 152].

**Morphological Characterization of LNCaP spheroids in PEG hydrogels**

Next, we examined the morphology of LNCaP cells grown for up to 28 days in 2% PEG hydrogels. Confocal images of Phalloidin-DAPI stained hydrogels (Figure 3.2B top panel) and timelapse videomicroscopy (Supplementary Movie 3.1) show that as cells proliferate in the hydrogel, they form multicellular mass. These colonies resemble the morphology of
Figure 3.2. Characterization of LNCaP cell growth cultured within PEG hydrogels. (A) Growth curves of LNCaP cells over 28 days for 3D cultures and 7 days for 2D cultures (mean ± SE) and live-dead staining at day 24 of LNCaP cells grown in hydrogels of different PEG content. The growth profile of cells is dependent on the PEG content, where growth rate is highest in 1.5% followed by cells grown in 2% hydrogels (left). No cell proliferation is detected in the stiffer hydrogels (2.5%). In 2D cultures (right), cells proliferate much faster reaching confluency within 7 days. Live-dead stainings (below 3D proliferation curve) reveal that most cells are viable (green) in both 1.5 and 2.0% hydrogels but not viable (red) in 2.5% hydrogels. Images were taken with CLSM (10x, 0.4 NA). Bright field images also confirm that cells in 2.5% hydrogels did not form colonies as observed in 1.5% and 2.0% hydrogels at
LNCaP colonies cultured in Matrigel™ as shown by us (Supplementary Figure 3.3) and others [7, 153]. The colony size increases from day 7 to day 21 and remains constant thereafter (Figure 3.2B bottom panel). As for their morphology, the colonies are spherical in shape up to day 21, then they become irregular as confirmed by a significant decrease in shape factor at day 28 (Figure 3.2B). The Phalloidin-Dapi and haematoxylin and eosin (H&E) stainings of frozen sections (Figure 3.2C) reveal that the day 28 colonies form ‘finger-like’ structures and often present a hollow central core. The presence of apoptotic cells in this region, as detected via Caspase 8 staining is also observed. This indicates an increased number of apoptotic cells within the core which has been also described in other in vitro 3D tumor cultures [72, 97]. Further histological examination of the hydrogel supports that the increase in size of each colony is proportionate to the cell number and size of cores formed (Figure 3.2D). Core formation is more frequently observed in day 28 cultures compared to the other time points (data not shown).

Comparison of LNCaP cell phenotype between 2D and 3D cultures in normal growth media

Since most of the previous Characterization of the LNCaP cell phenotype is based on 2D cultures, we set out to examine the 3D cultured LNCaP cell behaviour. As seen in Figure 3.3, LNCaP cells on 2D are spread out with
Figure 3.3. Immunostaining of histology sections comparing cell phenotype of 2D (day 6) and 3D (day 28) cultures grown in normal growth media. (Top) Cytoskeletal organization (actin, red) of cells in 2D cultures reveals a spread out and elongated morphology. The cell and nucleus (blue) size of 2D cultures is larger than the 3D cultures. In 3D cultures, cells adopt a cobblestone morphology and compact cell arrangement. They also form extensive cell-cell contact within the colony. (Middle) Epithelial marker, Ecad (green) is localised in the cell membrane and in cell-cell contacts for both cultures. (Bottom) Hypoxia staining by Pimonidazole (green) indicates that there is less hypoxic cells in 2D cultures relative to 3D cultures. CLSM images were taken at 40x magnification and 1.25 NA. Scale bars: 75 μm.
spindle-like morphology. In PEG hydrogels however, cells are arranged in a compact mass with a decrease in cell size relative to monolayer cells. Within the LNCaP colony, many cell-cell contacts that are localised with the epithelial marker E-cadherin (Ecad) are observed while in 2D cultures cell-substrata contacts are more prominent. We next performed a hypoxia staining with Pimonidazole in both cultures to evaluate the oxygen availability to cells, and detected higher hypoxia level in 3D than in 2D cultures. This implies that oxygen is less accessible to cells grown in 3D, due to the diffusion barrier imposed by the PEG hydrogel. Similar observations are also seen in clinical tumors, where hypoxia is a common feature in primary solid malignancies [154, 155].

Effects of R1881 on protein levels and gene expression in 2D and 3D cultures

During the initial stage of CaP, androgen availability and the androgen receptor (AR) signaling pathway play an important role in tumor progression [50, 156]. Therefore, androgen dependent LNCaP cells are widely used in 2D cultures for studying the AR/AR signalling which is activated by androgens and analogues such as R1881. In order to relate the already known LNCaP cell response to R1881 from 2D based experiments, we further investigated the effects of R1881 on CaP marker expression in 3D cultures (Figure 3.4). Immunofluorescent staining shows that AR localises to the cytoplasm of cells in both non treated 2D and 3D cultures but prominent nuclear stains were detected upon R1881 treatment in 2D cultures. On the contrary, AR is found mostly in extranuclear regions with weak AR stains in treated 3D cultures. Translocation of AR to the nucleus indicates activation of AR upon addition of R1881. This is also evident with an increase in the PSA (also known as kallikrein 3, KLK3) stainings observed in both 2D and 3D when stimulated with R1881. Localisation of the luminal epithelial marker CK8 is not affected by the treatment in 2D or 3D cultures. However, interestingly a small population (10-14%) of the colonies from treated 3D cultures express CK8 only in the centre of the colonies (Supplementary Figure 3.4).
Figure 3.4. Phenotypic comparison of LNCaP cell response to R1881 in 2D and 3D cultures. Immunostaining of histology sections compares the cell phenotypes of 2D (day 6) and 3D (day 28) cultures grown in androgen depleted media or R1881 supplemented media. Proteins of interest are in green, actin in red and nucleus in blue. (Left panel) In 2D cultures, AR is localised in the nucleus and PSA in the cytoplasm upon R1881 stimulation. Without treatment, the AR remains in the cytoplasm and PSA is not detected. The luminal epithelial
marker, CK8 is detected in both cultures regardless of treatment condition. In 2D cultures, CK8 clearly stains the filament fibers while in 3D, CK8 is localised at the cell border. (Right panel) In 3D cultures, extranuclear AR expression is found in both non treated and R1881 treated multicellular aggregates. PSA is also produced abundantly in R1881 treated 3D cultures but only at a very low level without when not treated. Magnified regions of each specific staining and culture condition (white boxes) are shown below the corresponding images. CLSM images were taken at 40x magnification and 1.25 NA. Scale bars: 75 μm and (magnified regions) 25 μm.

To examine the effect of R1881 on protein levels of AR, PSA and CK8, Western blotting was performed. Upon R1881 treatment, PSA and CK8 levels are enhanced with treatment in 2D and 3D cultures compared to non treated controls (Figure 3.5A, B). Interestingly, in treated 2D cultures, AR is significantly elevated when compared to 2D controls while no changes are detected in 3D cultures. Taken together, this suggests a differential AR protein expression in 2D and 3D cultures in response to R1881. The effect of R1881 on gene expression in 2D and 3D cultures was further investigated by qRT-PCR (Figure 3.5C). We found that PSA and CK8 mRNA levels are upregulated upon R1881 treatment in both cultures and reflect the protein changes upon treatment (Figure 3.5A, B). Similar to western blot analysis, AR mRNA expression in 3D cultures is also not affected by R1881 treatment while it is up-regulated in treated 2D cultures. In the absence of R1881, interestingly, PSA is significantly higher at mRNA level in the 3D cultures relative to 2D cultures. Expression of other androgen regulated genes was then further examined by microarray gene profiling.
Figure 3.5. Gene and protein expression of 2D and 3D cultures in response to R1881.
(A) A representative Western blot reveals an increase in production of all the investigated proteins in 2D cultures when treated with R1881 while in 3D cultures, only PSA and CK8 are elevated. (B) Signal ratio of protein relative to α-tubulin from Western blots of all treated and non-treated groups are presented as mean ± SE. The protein changes support the immunoblot in (A). Base level of PSA in non-treated 3D cultures is higher than non-treated 2D cultures. (C) qRT-PCR representing expression of LNCaP cell markers (mean ± SE) where PSA and CK8 are upregulated at the mRNA level upon treatment in both 2D and 3D cultures when compared to non treated 2D or 3D groups. The AR is only elevated in 2D cultures. Significant difference (p<0.05) between treated and non-treated samples within similar groups is denoted by (*) and between similar treatment of different groups are denoted by (Δ). Triplicate samples were analysed from at least three independent experiments.
Microarray analysis of differential gene expression in 2D and 3D cultures under the influence of R1881

We performed microarray analysis to comprehensively assess transcriptional differences between 2D and 3D LNCaP cell cultures and their response to the synthetic androgen R1881. Given the above described differences in PSA and AR expression and AR localization between 2D and 3D LNCaP cell cultures after R1881 treatment (Figure 3.4 and 3.5), we processed the microarray data as described in Materials & Methods to focus our analysis specifically on androgen-responsive genes. We found that 2D and 3D LNCaP cell cultures treated with R1881 share 2862 commonly regulated androgen-responsive genes (1165 up- and 1697 down-regulated genes) when compared to the respective ethanol controls. Surprisingly, the expression level changes of the majority of these genes were substantially reduced when R1881 was added to LNCaP cells in 3D culture when compared to their ethanol control in 3D culture (Figure 3.6A). Comparison of the 3D and 2D ethanol controls (E 3Dvs2D) revealed a strong androgenic response in the absence of R1881 in the 3D culture, showing differential regulation of 1469 androgen-responsive genes. 1180 of these genes were also commonly regulated in the R1881 treated 2D culture. This strongly suggests that the subdued response of androgen regulated genes to R1881 in the 3D culture (Figure 3.6A) was caused by a strong shift in the base line expression level of these genes when grown in 3D (Figure 3.6B). Notably, 349 (30%) of these genes displayed a higher fold change in expression levels in the 3D ethanol control when compared to the 2D culture after R1881 treatment. We examined these 1180 commonly regulated androgen-responsive genes (483 up- and 697 down-regulated genes) by pathway analysis with Ingenuity IPA software. In support of the strong androgenic response triggered by growing LNCaP cells in 3D culture, the IPA analysis ranked biosynthesis of steroids as the top canonical pathway based on the differential regulation of genes like farnesyl-diphosphate farnesyltransferase 1 a (FDFT1), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), isopentenyl-diphosphate delta isomerase 1 (IDI1), lanosterol synthase (LSS) and squalene epoxidase.
Figure 3.6. Microarray expression profile of androgen regulated genes in 2D and 3D LNCaP cell cultures. (A) Comparison of the expression levels of 2862 androgen-responsive genes commonly regulated in 2D (blue, 2D R1881vsE) and 3D (purple, 3D R1881vsE) LNCaP cell cultures after treatment with R1881. Genes were ordered on the vertical axis from highest to lowest fold change (log2) according to the 3D R1881vsE data. (B) Comparison of the fold changes (log2) in expression levels of 1180 androgen-responsive genes commonly regulated in R1881 treated 2D LNCaP cell culture (blue, 2D R1881vsE) and the 3D ethanol control (light blue, E 3Dvs2D). (C) Ingenuity Pathway Analysis (IPA) of the 1180 commonly regulated androgen-responsive genes. The network shows genes which are involved in lipid and steroid metabolism. Unbroken lines indicate direct interactions and broken lines indicate indirect interactions. The numbers and colors represent the fold change in expression levels (green=down-regulated and red=up-regulated) observed in the comparison of the ethanol controls of the 2D and 3D cultures (E 3Dvs2D).

(SQLE) (results not shown). Furthermore, the top networks listed by the IPA analysis are associated with lipid and steroid metabolism (Figure 3.6C). They contain node molecules like angiotensinogen (AGT), peroxisome proliferative activated receptor alpha (PPARA) and sterol regulatory element binding transcription factor 2 (SREBF2). Table 3.1
Table 3.1. Expression level changes of selected genes in 2D and 3D LNCaP cell cultures

<table>
<thead>
<tr>
<th>Gene</th>
<th>2D R1881vsE</th>
<th>3D R1881vsE</th>
<th>Ethanol 3Dvs2D</th>
</tr>
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<tbody>
<tr>
<td>KLK3</td>
<td>60.19</td>
<td>11.65</td>
<td>17.44</td>
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<tr>
<td>CXCR4</td>
<td>93.13</td>
<td>5.77</td>
<td>12.55</td>
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<tr>
<td>KLK2</td>
<td>129.29</td>
<td>27.21</td>
<td>8.98</td>
</tr>
<tr>
<td>NDRG1</td>
<td>18.79</td>
<td>4.33</td>
<td>6.57</td>
</tr>
<tr>
<td>TMPRSS2</td>
<td>36.78</td>
<td>18.39</td>
<td>3.51</td>
</tr>
<tr>
<td>HPGD</td>
<td>183.78</td>
<td>18.03</td>
<td>3.15</td>
</tr>
<tr>
<td>NKKX3-1</td>
<td>11.13</td>
<td>4.62</td>
<td>2.78</td>
</tr>
<tr>
<td>HSD17B2</td>
<td>47.09</td>
<td>2.99</td>
<td>1.77</td>
</tr>
<tr>
<td>FKBP5</td>
<td>90.43</td>
<td>55.73</td>
<td>1.74</td>
</tr>
<tr>
<td>THSD7A</td>
<td>24.23</td>
<td>5.36</td>
<td>44.77</td>
</tr>
<tr>
<td>STARSD4</td>
<td>6.79</td>
<td>2.41</td>
<td>3.14</td>
</tr>
<tr>
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<td>SREBF2</td>
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<td>2.69</td>
<td>2.16</td>
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<td>1.95</td>
</tr>
<tr>
<td>PPARA</td>
<td>2.44</td>
<td>1.55</td>
<td>1.75</td>
</tr>
<tr>
<td>AGT</td>
<td>-5.58</td>
<td>-1.64</td>
<td>-4.71</td>
</tr>
</tbody>
</table>

Expression level changes of classical androgen regulated genes (top) and members of the lipid and steroid biosynthesis pathway from Figure 3.7C (bottom) were derived from indicated comparisons between R1881 and ethanol (E) treated 2D and 3D LNCaP cell cultures.

illustrates the fold change in expression levels of a selection of classical androgen regulated genes and members of the lipid and steroid biosynthesis network in response to R1881 and culture conditions (2D and 3D). Altogether, these results show that growing LNCaP cells under androgen depleted condition in the 3D culture showed the hallmarks of a strong androgenic response and activated pathways responsible for cholesterol and steroid biosynthesis. Future experiments will address if factors like an increase in cell density and/or the number of cell-cell contacts (Figure 3.2-3.4) are responsible for the activation of the de novo synthesis of androgens in the 3D LNCaP cell culture.

Apart from steroid biosynthesis, IPA analysis also highlighted other top molecules, of which are associated with cell cycle, cellular movement, cell morphology and cell-to-cell signalling and interaction. Similar to the androgen responsive genes, non androgen responsive genes that play a role in cell-cell signalling and interaction, tissue development, cellular assembly and organization are also differentially expressed in the 3D and 2D cultures.
(Figure 3.7). In the 3D culture, there is a higher transcription of enzymes involved in matrix remodelling, particularly the lysyl oxidase (LOX). Genes that encode for ECM protein (collagens, e.g COL12A1) increase in their expression levels in 3D cultures compared to 2D cultures and vice versa for the basement membrane proteins (laminins e.g LAMA3). There is also a higher expression of genes such as neurogeliin 1 (NLGN1) and neurexin 1 (NRXN1), suggesting a greater cell differentiation capacity in the 3D microenvironment. As anticipated in the 2D cultures, the stiff surface induces an elongated cell morphology as also shown previously (Figure 3.3 and 3.4) and this is confirmed with the expression level of integrin alpha 2 (ITGA2), laminins, and mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminytransferase (MGAT5). Lower expression levels of genes related to focal adhesion formation such as vinculin (VCL) and Rho-associated, coiled-coil containing protein kinase 2 (ROCK2) in 3D cultures also indicate that LNCaP cells cultured in the softer hydrogel matrix is less likely to form focal adhesions compared to the stiffer 2D glass surface. Our findings reflect the cellular changes of cells from 2D cultures to 3D cultures as also reported by others [59, 62, 157-159].
Figure 3.7. Heat map of gene expression level comparing untreated 3D cultures to 2D cultures. The fold change in expression levels of genes related to cellular functions are represented by the colours in the heatmap. The green colour denotes a decrease in expression in 3D cultures relative to 2D cultures and the red colour denotes an increase in 3D cultures relative to 2D cultures. The fold change between -1.5 to +1.5 is considered not significant (NS). The differential expression of these molecules suggests that LNCaP cells grown in 3D may assume different roles in cell-cell signalling and interactions from the 2D cultures.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Biological functions</th>
</tr>
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<tbody>
<tr>
<td>ADAM9</td>
<td>Cell-matrix interaction/matrix remodelling</td>
</tr>
<tr>
<td>CD9</td>
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3.4 Discussion

The realization of the importance of the cell microenvironment in modulating cell signaling, migration and differentiation amongst other cellular physiology has led to the utilization of 3D in vitro cultures for cancer studies. Evidence from previous studies shows that 3D cultures are by far more physiological compared to monolayer cultures. To date, one of the common approaches used in 3D models is by embedding cells in either natural or synthetic ECM hydrogels. The advantage of synthetic hydrogels over natural hydrogels is that the biophysical and biochemical properties can be tailored independently. Though still in its infancy, the synthetic hydrogel is emerging as a potential substitute for the natural hydrogels frequently used in cancer research. In this study, LNCaP cells were cultured within the synthetic PEG-based hydrogel functionalised with RGD motifs and MMP cleavage sequences. Addition of these peptides provides adhesion sites for cells and allows matrix degradation by MMP activity, in which mimics the in vivo condition. Through characterization of the PEG hydrogels as well as the cell behavior, we are able to establish a 3D culture system that allows tumor-like morphogenesis. This study also presents for the first time phenotypic and genotypic comparison of LNCaP cells cultured within PEG hydrogels and on 2D tissue culture plastic.

Matrix properties (composition, stiffness) have been shown to influence the migration, morphology and growth of cells [59, 60, 160-163]. In PEG hydrogels, the matrix stiffness can be tuned by altering the PEG content, which allows us to profile the growth of cells cultured in three different matrix stiffnesses. As anticipated, the increase in PEG content accordingly increases matrix stiffness and diffusion hindrance, which is consistent with earlier studies by Ehbar et al. [142]. We have observed a significant effect of the matrix stiffness on cell growth. While cells in softer matrix (1.5% PEG, 1 kPa and 2.0% PEG, 4kPa) are able to proliferate faster, in stiffer matrix (2.5% PEG, >7kPa), growth is impeded. We rule out nutrient deficiency as the reason for non growth in 2.5% hydrogels as our study has shown that molecules of molecular weight up to 66 kDa, a range that encompasses most growth factors, can penetrate the PEG hydrogels.
Moreover, cells close to the surface of the 2.5% hydrogel are not able to grow despite having full access to nutrients. In another study, Bott et al. (2010) demonstrated that dermal fibroblasts can be cultured in the 2.5% hydrogel [109]. Therefore, the rigidity of the matrix itself may be the key regulator for cell growth. For 3D culture experiments, 2% PEG hydrogel was used. These hydrogels not only resemble the tissue stiffness of in vivo glandular tissues (4 kPa) and other soft tissues [56, 164] but also are compatible for LNCaP cell growth in a manner that recapitulates the slow growing tumor.

Many past studies confirm that there are vast phenotypic and genotypic differences between cells cultured in 2D and 3D since the stiffness of the substrata, geometry and cell-matrix interaction are highly different between the two models [6, 10, 59, 62, 135, 162, 165-168]. Our study shows that phenotypically, LNCaP cells form organised compact masses in PEG hydrogels. These colonies have central hollow cores and are apoptotic. Apoptosis has been shown to be implicated in formation of acini-like structures for glandular tissues. Normal breast epithelial cells MCF10A cultured in 3D Matrigel undergo cell polarization and eventually apoptosis to form hollow lumen [169]. Over the 28 days of culture in 2% hydrogels, a clearing of cells is observed in the core of the LNCaP colony as the colony size increases. Interestingly, the size of the core is also dependent on the size of the colony, suggesting that core formation could be related to nutrient deficiency. Additionally, loss of matrix attachments experienced by cells in the centre may also elicit metabolic impairment that leads to apoptosis [170]. All these features which resemble characteristics of avascular tumors, however, are not present in 2D cultures.

In the 3D culture, LNCaP colonies become irregular with multiple ‘finger-like’ structures as culture progresses to 28 days. Similar ‘fingering’ phenomena was also described by Anderson et al. where adaptive tumor cells in spheroids acquired the fingering structure under nutrient or oxygen depleted circumstances [171, 172]. In this case, the larger spheroids may require more nutrients and oxygen for their continual growth. Simultaneously, the high density cellular mass could also restrict nutrient diffusion leading to apoptosis as observed in the core of aggregates. Molecule exchange in the
hydrogels may not be efficient enough to sustain the nutritional or oxygen need of the spheroids. Evidence from the hypoxia marker also reveals that cells are hypoxic on day 28 of 3D cultures. Therefore, the irregular shaped spheroids could be a response of cells to this microenvironment stress. This could also be related to the gain of an invasive trait of spheroids when they are grown for a longer time [7], suggesting that cells could become more invasive in a less optimal condition. Such occurrence mimics clinical situations, as tumor cells are known to invade other sites or induce angiogenesis when the primary sites fail to sustain their growth.

In order for cells to invade, cells go through EMT and break down matrix barriers to allow cell migration. The gene expression analysis shows higher expression of EMT related genes and lower expression of epithelial markers in LNCaP cells cultured in PEG hydrogels compared to 2D cultures. This suggests that these cells may possess a more migratory phenotype in 3D compared to monolayer cultures. An up-regulation of matrix degrading enzymes, MMP3 and MMP13 in 3D cultures further supports this conclusion. Formation of ‘finger-like’ structures (Figure. 3.1D) also resembles collective migration occurring during neoplastic progression [173]. This could also be attributed to the cell-cell and cell-matrix interaction that modulate cell adhesion and morphological changes. Clearly, these interactions differ between the two cultures as shown in the differential expression of ECM proteins and integrins subunits.

Expression of common androgen regulated genes also differ between the two models, As shown in the qRT-PCR and microarray gene profile, baseline expression of positive androgen regulated genes (e.g. KLKs, lipid/steroid metabolic enzymes) in the absence of androgen is higher in 3D cultures than 2D cultures. An increased expression was also recently observed in 2D cultures with high cell density as compared to low cell density (unpublished). A similar observation was also reported where increase in baseline expression of the androgen regulated gene, PSA was also reported in a previous study where the PSA protein and transcripts were higher in the more metastatic LNCaP derived subline, C4-2 cells, compared to the parental line, under androgen-starved condition [174]. It is evident that the LNCaP cells under different microenvironments receive different biochemical
and biophysical cues from the surrounding ECM and neighboring cells, possibly promoting a more androgen independent phenotype. However, our microarray gene expression analysis does not show similar differential gene expression as reported by other studies of androgen dependent LNCaP cells and its castrate resistant subline, C4-2 [175, 176]. The PEG hydrogels could be used in future investigations of morphogenesis and development by assessment of phenotypic and molecular changes of castrate resistant prostate cancer cells in this system. Such an established model that represents the advanced stage of CaP would greatly contribute to translational studies of the disease and ultimately aid in identification of key factors (cell signalling pathway, biomarkers etc.) that trigger the progression to advanced CaP.

Stimulation with R1881 also reveals differential expression of AR at the protein and transcription level. This finding and the lack of nuclear AR translocation shown in immunofluorescent examination of LNCaP colonies, indicate that the transport of AR into the nucleus is less prominent in 3D cultures. The lack of nuclear translocation of AR in 3D cultures after R1881 stimulation however does not mean that AR signalling is not activated as androgen responsive PSA expression is clearly up-regulated. The intracellular interaction within the colony itself could render differential AR activation (transportation of AR). Studies have shown that integrins and cell attachment can influence AR activity [177, 178]. This could also be related to altered AR co-regulator recruitment, which potentially changes AR transportation [179]. Negative nuclear AR staining in this study could be a result of enhanced AR turnover; hence, AR protein is not elevated in 3D cultures despite the presence of R1881.

Discrepancies in AR protein and transcriptional levels are also apparent between 2D and 3D cultures when compared to their controls, where AR is elevated with R1881 treatment in 2D cultures but not affected in 3D. These observations are not consistent with other 2D based studies. Previously, it was reported that androgens down-regulate AR expression at the transcriptional level but increase AR protein expression [180, 181]. Nevertheless, a contradictory report by Wolf and colleagues revealed no change in protein levels after androgen stimulation [182]. This unexpected
AR expression pattern in 3D cultures could be attributed to the microenvironmental factors or phenotypic divergence of the cells in aggregates from the monolayer cells. Due to the cell density and size of the colony, the individual cell may receive a different dose of R1881 within an LNCaP colony. According to Kwok and Twentyman, they described that the growth rate and sensitivity to cytotoxic drugs are higher in the outer region of spheroids [183]. Hence, in this study, LNCaP cells at the periphery could be more sensitive to R1881 than cells towards the centre which resulted in a biased response between the two regions.
Chapter 4: Interactions between human osteoblasts and prostate cancer cells in a novel 3D \textit{in vitro} model
Chapter 4: Interactions between human osteoblasts and prostate cancer cells in a novel 3D in vitro model

Abstract

Cell-cell and cell-matrix interactions play a major role in tumor morphogenesis and cancer metastasis. Therefore, it is crucial to create a model with a biomimetic microenvironment that allows such interactions to fully represent the pathophysiology of a disease for an in vitro study. This is achievable by using 3D models instead of conventional 2D cultures with the aid of tissue engineering technology. We are now able to better address the complex intercellular interactions underlying prostate cancer (CaP) bone metastasis through such models. In this study, we assessed the interaction of CaP cells and human osteoblasts (hOBs) within a human tissue engineered bone construct (hTEBC). Consistent with other in vivo studies, our findings show that intercellular and CaP cell-bone matrix interactions lead to elevated levels of matrix metalloproteinases, steroidogenic enzymes and the CaP biomarker, prostate specific antigen (PSA); all associated with CaP metastasis. Hence, it highlights the physiological relevance of this model. We believe that this model will provide new insights for understanding of the previously poorly understood molecular mechanisms of bone metastasis, which will foster further translational studies, and ultimately offer a potential tool for drug screening.
4.1 Introduction

In North America, it is predicted that CaP will be the most diagnosed male cancer by 2009, accounting for more than 25% of new cancer cases [132, 184]. One of the most rampant complications of advanced CaP is bone metastasis. In high incidence countries such as the United States and United Kingdom, only 28-33% of men with metastatic disease survive up to five years [1, 185]. Management of this aspect of the disease is difficult, primarily due to insufficient knowledge about the cellular and molecular mechanisms involved in osteotropism of CaP cells. Numerous 2D culture studies and *in vivo* studies have been actively employed to further understand the complex mechanisms [186-189].

In a 2D culture, cells are not surrounded by a 3D microenvironment consisting of an ECM as it is in native tissues. The ECM provides biological and physical support to cells, and is involved in the modulation of cell behavior. On 2D surfaces, the lack of cell-matrix interaction causes disparity from the *in vivo* physiological situation which leads to changes in cell phenotypes and gene expression [190, 191]. These inconsistencies may result in less reliable experimental findings; therefore may not be translatable for clinical applications.

*In vivo* studies involving animal models no doubt offer more relevant clinical values; however, these models also have their limitations. This is because reproducibility is compromised, and pathology and disease progression in humans may not be represented accurately in animal models. Moreover, animal hosts add complexity to a study by contributing to multivariable factors (e.g. immune system). Such complications in *in vivo* models have lead to the development and utilization of 3D *in vitro* models.

3D *in vitro* models are able to compensate for the shortfalls of 2D cultures and *in vivo* models. In these models, cells are embedded within matrices resembling the natural ECM which allow cell-ECM interaction in a less complex and more well-defined condition. Previous studies using 3D models have shed light on phenotypic changes, cell signaling, angiogenesis and chemoresistance of cancer cells [72, 97, 141]. As we foresee the great utility of having such a 3D culture system for studying CaP-mediated bone
metastasis, we have established a novel 3D in vitro model using tissue engineering strategies to create a biologically competent bone-like microenvironment.

Our approach for this model is to recapitulate the condition first encountered by CaP cells metastasizing to bone, an environment featuring dynamic cell-cell interaction within a bone-like ECM. In order to do this, we cultured CaP cells within hTEBCs, fabricated using a human osteoblast (hOB) cell sheet-based technique. The hTEBC mimics the natural bone microenvironment in terms of architecture as well as ECM components [115]. We then further assessed the interaction between CaP cells and hOBs using this model by evaluating the expression of enzymes involved in metastasis and androgen production. This application is described herein.

Data collected from this study provides some insights into cellular responses in relation to cell-cell communication within the CaP cell-bone metastatic microenvironment, which may not have been ascertained from previously utilized models. Therefore, the established 3D model offers promise for the study of the mechanisms for bone metastasis, as well as having the potential to be a valuable pre-clinical tool particularly for drug development studies.

4.2 Materials and methods

Culture of CaP cell lines
PC3 and LNCaP cell lines were purchased from the American Type Culture Collection (ATCC). Both cell lines were cultured in RPMI growth media, comprising of RPMI media (Invitrogen), 10% fetal bovine serum (FBS, Thermo Scientific) and 10% penicillin/streptomycin. In all experiments, cells from passage 18-30 were used. PC3 cells are androgen independent cells that form osteolytic lesions while LNCaP cells are androgen dependent cells that form osteoblastic lesions.

Isolation and culture of hOBs
hOB explants were obtained from patients undergoing knee replacement surgery as approved by the institutional ethics committee of Queensland
University of Technology. Bone was collected, minced and washed with phosphate buffered saline (PBS) at least five times to remove connective tissues, adipocytes and other debris. Then, the bone pieces were treated with 0.5% trypsin for 15 min at 37 °C. After trypsinization, the bone pieces were washed and transferred to T175 tissue culture flasks. The bone explants were then maintained in α-MEM growth media. The α-MEM growth media consists of α-MEM media (Invitrogen), 10% FBS and 10% penicillin/streptomycin. Cells no later than passage five were used for all experiments.

Fabrication of hTEBCs
As described previously by Zhou et al. (2007) [12], we seeded 1x10^5 hOBs suspended in 20 uL growth media on 1.5 mm thick, 5 mm x 5 mm medical grade polycaprolactone tricalcium phosphate (mPCL-TCP) scaffolds (Osteopore, Singapore) in six-well plates. The pre-seeded scaffolds were incubated at 37°C for 90 min before the addition of 2 mL of α-MEM growth media. After the hOB culture reached 80% confluency in the wells, the media was changed to osteogenic media, consisting of α-MEM growth media supplemented with 10 mM glycerol 2-glycerophosphate, 50 μg/mL L-ascorbic acid-2-phosphate and 100 nM dexamethasone (Sigma). After three weeks of culture in osteogenic media, mineralized hOB sheets were formed. The sheets were detached from the plate surface with a cell scraper and wrapped around the pre-seeded scaffolds. Wrapped scaffolds continued to be cultured in osteogenic media until ready for seeding with CaP cells.

Direct co-culture of CaP cells and hOBs
For direct co-cultures, 5x10^5 CaP cells/20 uL (PC3 or LNCaP cells) were seeded on hTEBCs. CaP cells seeded on mPCL-TCP scaffolds were CaP cells control group. After seeding, the scaffolds were incubated for 90 min at 37°C to allow CaP cells to attach. After incubation, 1 mL of RPMI growth media was added and cultures were maintained until termination. hTEBCs prepared from the same hOBs source (same patient) were used for co-culturing with CaP cells and served as the hOB control group.
To prepare for analysis of MMP activity, co-cultures and control groups were cultured in RPMI growth media for 14 days after seeding of CaP cells on TEB constructs or scaffolds. They were then cultured in serum-free RPMI media for 48 hours. The serum-free conditioned media (CM) was then collected to be analyzed for MMP activity. Regarding the study of steroidogenic enzymes expression, LNCaP cells and hOBs were co-cultured in RPMI growth media for 14 days. The growth media was then changed to androgen-depleted media (RPMI with 5% charcoal stripped serum) and cultured for 96 hr with media change at 48 hr interval. Cells were then harvested for RNA isolation.

**Microscopy image analysis of co-cultures**

Investigation of the exterior structure of hOBs and CaP cells, and constructs was performed using scanning electron microscopy (SEM). The hTEBCs and co-cultures were fixed with 3% glutaraldehyde then washed thoroughly with cacodylate buffer. The samples were treated with 1% osmium tetraoxide before being dehydrated sequentially with ethanol solutions. In order to preserve their surface detail, they were air dried by evaporation of hexamethyldisilazane (HMDS) before sputter coated with gold using Biorad SC500. Viewing and analysis were performed using the Quanta 200 Scanning Electron Microscope (FEI).

For visualization of CaP cells and hOBs in a co-culture, a wide spectrum cytokeratin, pan CK and cytoskeletal fluorescent stainings were used to distinguish the two cell types. Co-cultures and control groups were fixed with 4% paraformaldehyde (PFA) for 20 minutes and treated with 0.2% Triton-X for 15 min. Samples subjected to cytoskeletal staining were incubated with 0.8 U/mL rhodamine-conjugated Phalloidin (Invitrogen) for 45 minutes and DAPI for 40 minutes. Pan cytokeratin immunofluorescent staining involved incubation with diluted (1:300) mouse anti-human pan cytokeratin (pan CK) antibody (Abcam) for one hour. Following that, the samples were incubated with Alexa Fluor® 488 goat anti- mouse IgG antibody (1:300) for one hour. Finally, they were counterstained with cytoskeletal stain. Fluorescent images of 100-200 μm z stacks thick were captured using Leica SP5 CLSM (Leica) and presented as overlaid images.
Gelatin zymography of co-cultures

The activity of MMP2 and MMP9 was analyzed using gelatin zymography. CM collected from co-cultures and control groups were quantified with bicinchoninic acid (BCA) assay according to supplier's instructions (Pierce). A 12% SDS-polyacrylamide gel containing 1mg/mL gelatin was prepared. From the CM, 5 ug of protein were mixed with 5x loading buffer (0.05g bromophenol blue, 5 mL glycerol, 1 g SDS, 1 M Tris, pH 6.8) and loaded into the prepared gels. The gels were electrophoresed at 120 V for 2 hours at 4°C. Once the electrophoresis was terminated, the gels were removed and washed once with 2.5% triton-X for 30 min. The gels were then incubated for 1 hour in the same solution on a shaker. Gelatinolytic reaction was induced by incubation at 37°C in a buffer containing 50 mM Tris, 10 mM CaCl and 50 mM NaCl2 for 24 hours. The gels were then stained with 0.25% Brilliant Blue R250 and destained with 9% acetic acid and 36% methanol solution for approximately one hour or until apparent gelatinase activities were detected.

RNA isolation and quantitative Real-Time PCR (qRT-PCR)

A qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) to analyze expression of enzymes involved in the steroidogenic pathway for the co-culture and control groups. The RNA of cells from direct co-cultures and control groups was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. Then, the RNA was quantified using the ND-1000 NanoDrop spectrophotometer (NanoDrop, Delaware). 1 μg of RNA was treated with DNase I Amp grade (Invitrogen) before being reverse transcribed to cDNAs with Super Script III (Invitrogen). Changes in expression of steroidogenic enzymes, Cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1), Hydroxysteroid (17-beta) dehydrogenase 3 (HSD17B3), Retinol dehydrogenase 5 (RDH5), PSA and Fatty acid synthase (FASN) were analyzed with SDS 2.3 software by means of the $2^{\Delta\Delta C_T}$ method [192]. Primers used were as follows: CYP11A1, 5’-AGTTTCTCGGGACTTCGTCAGT-3’ and 5’-GGAGCCCGCCTTCTTCTGA-3’, HSD17B3, 5’-TGGGACAGTGCCGAGTGA-3’ and
5'-CGAGTACGCTTTCCCAATTCC-3',
RDH5, 5'-GCCCGCCAGCAATGC-3' and
5'-CGCCCAAAGCCTGAGTCA-3', PSA, 5'-TCTGCGGCGGTGTTCTG-3' and
5'-GCCGACCCAGCAAG-3', and
FASN, 5'-CGCTCGGCATGGCTATCT-3' and
5'-CTCGTTGAAGACGCATCCA-3'.

Statistical analysis
ANOVA post hoc Tukey tests were performed to determine the statistical
significance of steroidogenic enzyme expression between hOB-LNCaP co-
cultures and control groups. By conventional criteria, the difference is
considered to be statistically significant when p<0.05. Four independent in
vitro experiments were conducted for each condition.

4.3 Results

Fabrication of hTEBC using cell sheet-based technique
We used a cell sheet-based technique to wrap mPCL-TCP scaffolds within
hOB sheets for fabrication of the hTEBC. This method has successfully
produced well characterized hTEBcs from alveolar osteoblasts and bone
marrow stromal cell [12, 193]. hOBs were isolated from explanted human
bone. hOBs from the bone tissue detached from the tissue and began to
grow onto the tissue culture surface (Figure 4.1A). These hOBs were
expanded and used for seeding onto mPCL-TCP scaffolds. Over time, the
pores of the pre-seeded scaffolds were gradually filled with proliferating
hOBs (Figure 4.1B). This was illustrated by cytoskeletal structures of hOBs,
forming a fibrous network across the struts as visualised by Phallodin-DAPI
staining. hOBs which did not attach to the scaffold during seeding continued
to grow in the tissue culture well and were maintained until they were
confluent before being subjected to osteogenic induction to form hOB sheets.
When the hOB sheets became highly mineralized and deposited bone matrix
proteins, the sheets were used to wrap the pre-seeded scaffolds (Figure
4.1C); these constructs are termed as hTEBC that mimic the bone
microenvironment. To determine whether the hOBs survive the mechanical
Figure 4.1. The structure and cell morphology of the hOB sheet-based hTEBC. (A) Bright field microscopy image of the bone explant culture shows hOBs growing from the bone to the tissue culture surface indicated by the black arrow. (B) CLSM fluorescence image of hTEBC stained with Phalloidin (cytoskeleton, red) and DAPI (nucleus, blue) at the pore region of the scaffold reveals gradual growth of hOBs to fill the pore of the scaffold. (C) SEM images of the scaffold completely wrapped within the hOB sheet. (D) CLSM fluorescence image of the hTEBC stained with FDA and PI demonstrates live hOBs (green) significantly exceed dead cells (red). (E) Bright field image corresponding to (D) illustrates the orientation of scaffold struts (white arrows, s) that provide support to the hOB sheet. CLSM images were taken at 10x magnification and 0.4 NA. Scale bars: (A) 100 μm, (B, D, E) 250 μm, (C) 2 mm.

intervention and change to a 3D environment, FDA-PI (live and dead) staining was performed. Evidently, more than 90% of hOBs were viable and remained intact within the hTEBC up to more than 40 days in culture (Figure 4.1D, E). These constructs were used for co-culturing with CaP cells.

Morphology of cells in co-cultures

The CaP cells (PC3 or LNCaP lines) were directly seeded on the hTEBC and cultures were terminated at day two and day six. By culturing these two CaP cell lines within the hTEBC, we are mimicking the osteolytic (bone degrading) and osteoblastic (bone forming) response observed in CaP-mediated bone metastasis demonstrated by PC3 and LNCaP cells respectively. Phalloidin
staining alone showed extensive cell proliferation but did not clearly distinguish between CaP cells and hOBs (Figure 4.2A). Use of a cancer epithelial-specific antibody against pan CK and Phalloidin staining allowed visualization of both the CaP cells and hOBs by distinguishing between cell morphologies as shown in Figure 4.2B.

CLSM examination of pan CK and Phalloidin stained day six co-cultures revealed differences in CaP cell morphology and the ability of PC3 and LNCaP cells to form clusters within the bone matrix (Figure 4.2B). In the hOB-PC3 co-culture, PC3 cells appeared spread out assuming a fibroblastic and spherical morphology reflecting a mixed population of epithelioid and mesenchymal PC3 cells as also reported by others [113, 194]. LNCaP cells on the other hand appeared more adherent to one another, which explains their loss of normal fibroblastic morphology (as seen in monolayer cultures). This also promoted formation of cell clusters in the hOB-LNCaP culture. These observations are consistent with SEM images of the co-cultures (Figure 4.3A, B).

In addition to cell morphology analysis, the exterior structure of the cells in the direct co-cultures was examined using SEM. From the two day old hOB-PC3 and hOB-LNCaP co-cultures, cell density of PC3 cells within the TEB (Figure 4.3A2) was lower compared to LNCaP cells (Figure. 3B2), perhaps indicating a difference in affinity between the two cell types towards bone matrix. LNCaP cells showed a higher integration into the bone matrix and formed cell clusters that merged into the multilayered mass (Figure 4.3B3). The mass appeared highly compact assuming a tissue-like structure; however, this appearance was not present in the hOB-PC3 co-culture (Figure. 4.3A3). Both the PC3 and LNCaP cell morphologies exhibited on SEM (Figure. 4.3A3,3B3) are similar to the day six co-cultures as observed with immunofluorescent staining (Figure. 4.2B).
Figure 4.2. Fluorescence images of direct co-cultures of hOBs and CaP cells within hTEBCs viewed using CLSM. (A) The 2 day old co-culture stained with Phalloidin (red) and DAPI (blue) shows fibrous network of hOBs scattered with round-bodied CaP cells (20x, 1.0 NA). (B) Immunofluorescent staining with anti-pan CK (green) and Phalloidin (red) of the day 6 co-culture reveals morphology of PC3 and LNCaP cells, and distinguishes hOBs from CaP cells (40x, 1.25 NA). PC3 cells are characterized by a spherical (white open arrow) and fibroblast appearance (white close arrow). LNCaP cells adhere to each other to form aggregates (yellow open arrow) while monolayer cells assume a fibroblastic morphology (yellow close arrow). Scale bars: (A) 250 μm and (B) 75 μm.
Figure 4.3. SEM images of direct co-cultures of hOBs and CaP cells within hTEBCs. The co-culture was harvested 2 days after seeding with PC3 (A1-3) and LNCaP (B1-3) cells. Circled regions denote magnified areas at 300x (A2,B2) and 1000x (A3,B3). The hOB-PC3 culture appears more homogenous and less dense (A2) when compared to the hOB-LNCaP culture which appears coarse and compact (B2). Within the hTEBC, LNCaP cells form multiple clusters (blue arrows) and layers that are integrated within the matrix (B3). Such features are absent in the hOB-PC3 culture (A3). Scale bars: (A1,B1) 2 mm, (A2,B2) 400 μm and (A3,B3) 100 μm.

MMP activity in co-cultures
Proteolytic enzymes such as MMP2 and MMP9, among other types of MMPs that degrade ECM components, have been implicated in cancer cell proliferation and migration [195-197]. To further investigate the effect of CaP cell-hOB interaction on MMP activity, gelatin zymography was performed. Activity of the MMP2 and MMP9 was detected as bands on zymograms (Figure 4.4A). MMP activity of the co-cultures was compared to the control groups comprising of LNCaP or PC3 cells cultured on scaffolds and the hOBs on the hTEBC alone. Both co-cultures and hTEBC control group showed the presence of pro-MMP2. While MMP9 activity was not detected in any of the control groups, it was clearly enhanced in the hOB-PC3 co-culture (Figure. 4.4A). Other groups have also reported augmentation of MMP9 both in vitro and in vivo with an increase in MMP9 activity when PC3 cells were cultured with bone explants as well as in a bone xenograft colonized by PC3 cells [187, 196]. Our findings from this in vitro study indicate association to the osteolytic nature of PC3 cells as proven in in vivo studies [129, 130].
Figure 4.4 Interactions between hOBs and CaP cells in direct co-cultures. (A) Zymograms of serum-free CM collected from the control and co-culture groups. MMP9 standard was used as the marker, while pro MMP2 was identified according to their protein size. MMP-9 is detected in CM of the hOB-PC3 co-culture and not in the hOB-LNCaP co-culture or the control groups. (B) Expression of enzymes involved in synthesis of DHT and CaP progression in the LNCaP cultures, the hOB cultures (hTEBC) and the hOB-LNCaP co-cultures under an androgen-depleted condition. Bars represent mean fold change at the mRNA level from LNCaP group ± SE for each enzyme. mRNA levels detected in hOB-LNCaP and hOB cultures were normalised against LNCaP cultures. Statistically significant difference (p<0.05) between all the culture groups is indicated by ( * ). RDH5, HSD17B3 and CYP11A1 are highly expressed by hOBs more than LNCaP cells. FASN and PSA expression in hOBs are negligible, however in the co-cultures expression of both enzymes is significantly higher than hOB and LNCaP controls respectively. Triplicate samples were analysed from four independent experiments.
**Expression of steroidogenesis enzymes in co-cultures**

In an attempt to relate to the clinical scenario, we used this 3D co-culture model to investigate the effects of the metastatic microenvironment on CaP cells that are reliant on external androgens for growth by co-culturing LNCaP cells (androgen dependent) and hOBs under an androgen-depleted condition to mimic the system of patients who have undergone androgen deprivation therapy (ADT). After culturing for 14 days, the growth media containing androgens was replaced by androgen-depleted growth media and maintained for four days in this “starved media”. Cells were then harvested for RNA extraction. Expression of several enzymes involved in steroid synthesis (Supplementary Figure 4.1) and the androgen regulated CaP biomarker, prostate specific antigen (PSA) were analyzed by qRT-PCR. (Figure 4.4B)

PSA is a biomarker for CaP progression and was significantly upregulated in the hOB-LNCaP co-cultures relative to LNCaP only controls (p=0.0108). It is important to note that PSA was not expressed by hOB controls (hTEBC) but was highly upregulated in presence of hOBs. This indicates that hOB-related factors may increase the aggressiveness of CaP cells. Fatty acid synthase (FASN), which is upregulated during CaP progression, did not increase in the co-cultures relative to LNCaP controls but was higher compared to hOB controls. This suggests that the influence of hOBs does not change the need for lipids as an energy source, nor is it likely to contribute to enhancement of that particular pathway.

As shown in Figure 4.4B, most of the steroidogenic enzymes were co-expressed by both LNCaP cells and hOBs. In particular, cytochrome p450 family member 11A1 (CYP11A1) and 11-cis-retinol dehydrogenase (RDH5) of hOB cultures were ten times higher in expression compared to LNCaP cultures (p=0.0419 and 0.0252 respectively, Figure 4B). Significantly, CYP11A1, the first enzyme committing cholesterol to steroidogenesis, was significantly upregulated in the co-cultures relative to LNCaP only controls (p=0.0004). In cases where expression of steroidogenic enzymes was higher in hOBs, there was also an increased expression in the co-cultures above that detected in LNCaP cells although lower than that detected in hOBs alone.
Other groups have also reported that hOBs express enzymes responsible for conversion of precursors to potent androgen [198-200]. Indeed, they suggest that the hOBs could play a role in the production of the potent androgen, dihydrosterone (DHT), which could sustain the survival of CaP cells. At this stage, we are unable to determine which cell type contributes more to the upregulation or downregulation of the genes expressed in the co-culture as both cell types could not be separated for analysis.
4.4 Discussion

With the growing acceptance of 3D in vitro models as tools for studying cancer biology, many other improved and novel 3D models have emerged in an attempt to recapitulate the native tumor and its microenvironment [6, 72, 120, 141, 201]. A current shortage of appropriate in vitro models to study CaP cells and hOBs interaction in relation to bone metastasis has compelled our group to employ a 3D in vitro model for this purpose. Thus, we have established a direct co-culture model to assess the cellular and molecular interactions between hOBs and CaP cells.

This 3D direct co-culture model consists of CaP cells cultured within an engineered bone-like microenvironment, reflecting the initial condition encountered by CaP cells metastasizing to bone. The hTEBC of this model to a certain extent mimics the bone microenvironment by providing the mechanical support to the CaP cells and surrounding them within the bone ECM, as well as contributing the necessary growth factors [115]. In addition, by using primary hOBs instead of modified hOB cell lines from other origins we have improved the biomimetic nature of the construct with respect to native human bone tissues. With controlled mechanical intervention throughout the wrapping of the scaffold, hOBs remain viable thereafter during the experimental period. The integrity of the hOBs is crucial at this point to allow a dynamic intercellular communication in the co-culture model.

From the direct co-culture model, apparent cell-cell and cell-matrix interactions are observed. These interactions illustrate the affinity of the CaP cells for the bone matrix and integration within this microenvironment. Such characteristics are influenced by the CaP cell phenotype as demonstrated by the osteoblastic LNCaP cells. In the hOB-LNCaP co-culture, LNCaP cells assimilate to the matrix to form a tissue-like mass. Judging from the compact structure, this may be reflective of the osteoblastic lesions known to be caused by LNCaP cells [202, 203] as observed in vivo by Yonou et al. (2001) [130] when LNCaP cells metastasized to human bone chips in Non-Obese Diabetic/Severe Combined Immunodeficient mice. Similarly, culturing of known osteolytic PC3 cells with hOBs resulted in an increase of MMP9 production and possibly MMP9 activation. This augmentation may be
associated with raised invasive potential of CaP cells as indicated by previous studies [204, 205]. These findings confirm the physiological relevance of this model for analytical assays and translational studies.

We have further explored the application of this model for other clinical conditions namely, post castrate metastasis. Prostatectomy causes androgens to decline to a minimal level, causing apoptosis of androgen-dependent CaP cells. However, Locke et al. (2008) [206] have reported that de novo synthesis of androgens, minimal before castration, is upregulated as tumors move towards castrate resistant. This could explain the recurrence of CaP in patients even after ADT. Here, we have utilized hOB-LNCaP co-cultures to mimic the post castration bone metastasis scenario in an androgen-depleted environment. The increase in CYP11A1 and RDH5 expression in hOB-LNCaP co-cultures suggest that synthesis of the potent androgen, DHT, from circulating precursors may occur in CaP bone metastasis. Simultaneously, high expression of steroidogenic enzymes in hOBs may contribute androgens or precursor steroids to drive CaP tumor growth and aggression, as indicated by a corresponding rise in PSA.

Though it is clear that CaP-hOB cell interactions influence each others’ mRNA expression profiles, we have yet to determine the signaling pathways or bioactive molecules leading to the aforementioned phenomena. Another question raised here is which cell type has initiated the mRNA alterations observed in the co-cultures. Nevertheless, we postulate that the three factors which could explain the resilience and progression of CaP cells during post castration bone metastasis are: hOBs are able to compensate for the lack of androgen; the CaP cells respond to the bone environment by producing more steroidogenic enzymes; and the synergistic upregulation of enzymes from both cell types could essentially contribute to de novo synthesis of DHT or other steroids. Since all the genes tested except PSA, are jointly expressed by both cells, the pooled mRNA reflects cumulative expression of the cell mixture. To overcome this issue in future experiments, we can resort to cell sorting or modifying the 3D model to enable more effective separation of the CaP cells and hOBs.

Although the physical CaP cell-bone interactions evidently impacted on the development and progression of bone metastasis, the biochemical
interaction between these cells is no less important. In fact, an indirect 3D co-culture model would be a great addition to the current model to investigate paracrine interactions between CaP cells and bone. Our study so far proves that this 3D direct co-culture model is both practical and versatile for studying intercellular and cell-matrix interaction at a cellular and molecular level. Hence, this model is a useful tool to elucidate the multistage process of bone metastasis or osteotropism of CaP cells. A biomimetic in vitro model is a critical asset for translational studies including drug target selection prior to clinical trials. Our approach is less demanding compared to in vivo models and yet is able to marry the complexity of native cellular physiology with the simplicity of in vitro systems.
Chapter 5: Utilization of a two-construct 3D *in vitro* model to study the paracrine interaction of prostate cancer (CaP) cells and human osteoblasts (hOBs)
Chapter 5: Utilization of a two-construct 3D in vitro model to study the paracrine interaction of prostate cancer (CaP) cells and human osteoblasts (hOBs)

Abstract

As microenvironmental factors such as 3-dimensionality and cell-matrix interactions are increasingly being acknowledged by cancer biologists, more 3D in vitro models are being developed to study cancer development. However, to understand the pathophysiology of bone metastasis, a more complex in vitro model is required. Here, we have established an indirect 3D co-culture model to investigate the paracrine interactions between CaP cells and hOBs. Co-culture of the human CaP cells (LNCaP) embedded within PEG hydrogels with human tissue engineered bone construct (hTEBC), resulted in reduced proliferation of LNCaP cells and suppression of Alkaline phosphatase (ALP) in hOBs. Importantly, we have proven that this model can also be used to examine the effects of androgen on the CaP-bone interaction. When the co-cultures were supplemented with the androgen analogue, R1881, an increase in the expression of androgen regulated genes, kallikreins (KLKs), Fatty acid synthase (FASN), was seen (mRNA and protein induction) in LNCaP cells. Interestingly, up-regulation of kallikreins is also detected in LNCaP cells co-cultured with hTEBC that did not receive R1881 treatment. From the mRNA and protein analyses, markers of LNCaP cells (Cytokeratin 8, E-cadherin) and osteoblasts (Vimentin, Osteocalcin, RUNX2) continue to be expressed. The cellular and molecular changes of LNCaP cells seen after co-culture with hTEBCs indicate that osteoblasts exert a paracrine effect on LNCaP cells and vice versa. Taken together, this model is a biological relevant model that can be used for many investigative studies pertaining to the intercellular crosstalk of CaP cells and bone.
5.1 Introduction

Prostate cancer is one of the leading cause of cancer death in men particularly in the western countries [132, 133]. Although current treatments are able to curb the disease at an early stage, however, curative approaches are currently less realistic once CaP progresses to a metastatic stage. In advanced CaP, at least 70-80% patients are also diagnosed with bone metastases which limit their survival to less than 30% within 5 years [185]. To date, while the general pathophysiology of bone metastasis is known, the specific cellular and molecular mechanisms that spur homing of CaP cells to bone sites to form bone metastasis are still not fully understood. Hence, it is crucial to unravel the complex interactions between CaP cells and the bone/bone environment. Direct or indirect interaction is evident from in vitro and in vivo studies as well as from histopathologic examination of clinical samples [110, 207-209]. Naturally, these bi-directional interactions play a major role in not just attracting the CaP cells to bone sites but also conditioning the cells to adapt to the new and less familiar environment. Numerous factors have been associated with providing fertile ‘soil’ for circulating tumor cells such as secreted growth factors (TGFβ, IGF, BMP, VEGF), cytokines (RANKL, interleukins, CXCL12) and bone matrix proteins (osteopontin, bone sialoprotein and collagen type I) [30, 45, 210, 211]. These factors are involved in CaP cell proliferation, chemotaxis and anchorage to the bone matrix.

Since CaP cells interact with bone under a dynamic milieu, composed of stroma cells, ECM and growth factors, it is difficult to imitate this condition in a 2D culture system. Nevertheless, 2D cultures are still widely employed for studying cell signalling pathways, characterization of metastatic CaP cells and their gene expression [111, 112, 212-214]. The common method used for studying the CaP-bone interaction is by indirect or direct co-culture of both cell types, separated by a transwell or mixed together, respectively in 2D cultures [110-112]. Considering the importance of the biochemical and physical properties of ECM in modulating cell behaviour and tumor progression, 2D cultures lacking these features poorly reflect the physiological condition [56, 63, 67, 136, 137]. Hence, translation of results to
human applications has to be carefully interpreted. *In vivo* models on the other hand better mimic the human disease progression in bone however, this complex system presents an obstacle for studying specific CaP-bone interaction without the influence of other intrinsic factors from the host. Furthermore, animal models do not fully represent the human CaP metastasis pathogenesis. Some of these limitations can be overcome by 3D *in vitro* culture systems, which can recapitulate the cell’s native microenvironment under a well controlled condition.

A small number of groups have successfully used 3D *in vitro* cultures in cancer research by embedding cells in either natural or synthetic matrices, and liquid-overlay approaches to generate multicellular spheroids [6, 7, 201]. These 3D *in vitro* cultures have significantly restored the functional differentiation, structural organization and migration mode of cells which are lost in monolayer cultures [215, 216]. In addition, 3D cultures also allow heterotypic culture of different cell types e.g. cancer cells co-cultured with osteoblasts, bone marrow derived cells, fibroblasts and endothelial cells [102, 103, 105, 119, 217]. Previously, we and others have demonstrated that tissue engineered bone can be used as a proof of principle to answer biological questions related to bone metastasis [118, 127, 131, 218]. In particular, our group uses medical grade polycaprolactone-tricalcium phosphate (mPCL-TCP) scaffolds to fabricate human tissue engineered bone constructs (hTEBC), which have also been used for the past 4 years as cranial implants on human patients [11]. However, from our earlier study (chapter 4), when CaP cells were directly cultured with the hTEBC, we encountered difficulty in separating the CaP cells and hOB for analysis. Therefore in this study, we used a 3D *in vitro* model consisting of two attached constructs: LNCaP cells embedded in synthetic PEG-based hydrogels and hTEBC. Both constructs provide 3D mechanical support to cells and matrix stiffness that approximate each cell types’ native microenvironment.

Herein, we aim to establish a reproducible 3D co-culture model that can be effectively used for investigating the paracrine interactions between LNCaP cells and human osteoblasts (hOBs). As androgens play a dominant role in gene regulation, survival and progression of CaP cells [219-223], we
set out to examine the influence of CaP-hOB interaction on the effects of synthetic androgen, R1881 on LNCaP cells. In this study, we observe that the presence of hTEBC influences the proliferation of LNCaP cells and their gene expression in response to R1881. It also provides proof of principle that a CaP-hOB paracrine interaction can be forged in this tissue interface in vitro model. Taken together, we have presented a novel 3D in vitro model that has the potential to dissect specific biological hypotheses related to extensive genomic or proteomic assessments to further our understanding of the CaP-bone crosstalk.
5.2 Materials and methods

Materials
Medical grade polycaprolactone tricalcium phosphate (mPCL-TCP) sheets were purchased from Osteopore Singapore. The sheet was trimmed to 5x5 mm size and 1.5 mm thick scaffolds. The synthetic androgen, R1881, was purchased from DuPont. For immunostaining and western blotting, primary antibodies against Vimentin (Developmental Studies Hybridoma Bank), Androgen receptor (AR) terminal C-19 (Santa Cruz), Cytokeratin 8 (CK8) M20 (Abcam), 11-cis-retinol-dehydrogenase (RDH5, Abnova), Fatty acid synthase (FASN, Santa Cruz) and PSA (Dako Cytomation) were used.

Cell culture
Androgen dependent LNCaP cell lines were purchased from American Type Culture Collection (ATCC). Cells were maintained in RPMI growth media, comprising of phenol red–free RPMI media (Invitrogen), 10% fetal bovine serum (FBS, Thermo Scientific) and 1% penicillin/streptomycin. Cells from passage 22-28 were used in all experiments. Primary human osteoblasts (hOBs) were isolated as described in Sieh et al. (2010) [131] (chapter 4). The isolated hOBs were cultured in hOB growth media consisting of α-MEM media (Invitrogen), 10% FBS and 1% penicillin/streptomycin.

Preparation of PEG hydrogels
PEG-based hydrogels were prepared as previously described by Ehbar et al. (2007) and our group [108, 109, 142]. In summary, PEG-Gln/PEG-MMP-Lys precursor stock solution (5% w/v) was diluted to the desired PEG concentration in Tris-Buffer (50 mM, pH 7.6) containing 50 μM RGD conjugate, and 50mM calcium chloride. Then, immediately after adding 10 U/mL thrombin-activated factor XIII, LNCaP cell suspension was added into the reaction mixture to yield a final cell density of 3.5x10^5 cells/mL, and hydrogel discs were formed by sandwiching 25 μL drops of the reaction mixture between two sterile glass slides (separated by 1.5 mm spacers) pre-coated with SigmaCote (Sigma). Hydrogels were allowed to polymerize at 37
˚C in a humidified incubator for 30 min before being transferred to 24 well plates filled with growth medium. Medium was changed every 4 days.

**Preparation of human tissue engineered bone construct (hTEBC)**

Using the same method described by Sieh *et al.* (2010) [131] (chapter 4), the hTEBC was prepared from hOBs. Osteogenic media used to induce mineralization of hOB matrix was prepared by supplementing α-MEM growth media with 10 mM glycerol 2-glycerophosphate, 50 μg/mL L-ascorbic acid-2-phosphate and 100 nM dexamethasone (Sigma). hOBs cultured in osteogenic media formed mineralized hOB sheets that were eventually used to wrap around the pre-seeded scaffolds. Wrapped scaffolds continued to be cultured in osteogenic media until ready to be co-cultured with LNCaP cells.

**Preparation of LNCaP-hTEBC indirect co-culture and R1881 treatment**

After hydrogels embedded with LNCaP cells were prepared, they were attached to the hTEBC by the same hydrogel material with 1.5% PEG content without addition of RGD motifs. The glue consisted of the hydrogel mixture (1.5% PEG) without RGD. After loading 4 μL of the reaction mixtures onto the hydrogel, immediately, the hTEBC was placed on top of the hydrogel. The biphasic construct was incubated for 20 min at 37 °C to allow the glue to polymerize. The co-culture was maintained in LNCaP growth media for 28 days for proliferation analysis. As for R1881 treatment, after 24 days in LNCaP growth media, media was changed to hormone deprived media (RPMI media with 5% charcoal stripped serum). After 48 hours in hormone deprived media, co-culture were maintained for further 48 hours in similar media supplemented with R1881 (1 nM). For non treated controls, cells were cultured in hormone deprived media with 0.0008% ethanol.

**Proliferation assay**

The hydrogel control and hydrogel from co-culture maintained in LNCaP growth media were harvested at day 1, 7, 14, 21 and 28. The hydrogel and hTEBC were separated from the co-culture construct. The samples were digested with 300 μL of Proteinase K for 16 h at 56°C. Thereafter, the Pico green assay was performed according to manufacturer's instructions. The
digested cell suspension mixture was diluted with PBE buffer to allow readout within the range of the standard curve between 2μg/mL and 10ng/mL DNA. All samples were assayed in triplicate.

**Alkaline phosphatase (ALP) assay**

Cells were lysed with 0.2 M Tris buffer containing 0.1% Triton X-100 by vigorously suspending the media and incubating the samples at room temperature for 15 min. Once cells were lysed, lysates were pelleted and 100 μL of supernatant transferred to 96 well plates. The ALP assay is based on the dephosphorylation of para-nitrophenyl phosphate (pNPP) substrate by the ALP that produces colorimetric change. pNPP substrate (SigmaFAST™, Sigma) was prepared according to manufacturer’s instruction. 200 μL of pNPP substrate was then, added to sample supernatant. The plate was incubated in the dark for 30 min at RT and absorbance was measured at λ=405 nm. Total protein of the sample supernatant, which was collected earlier, was quantified with the bicinchoninic acid (BCA) assay according to supplier’s instructions (Pierce).

**Microscopy image analyses**

**Phalloidin-DAPI staining**

Cells from monocultures or co-cultures were fixed with 4% formaldehyde/PBS and permeabilised with 0.2% Triton X. This was followed by incubation in 0.8 U/ml rhodamine conjugated Phalloidin (Invitrogen) and 2 μg/ml 4’,6-diamidino-2-phenylindole (DAPI) [Invitrogen] solution for 40 min as described previously [131]. Fluorescent confocal images stacks over a range of 100-200 μm were captured with Leica SP5 CLSM. From the 3D projections, the size and shape factor of the colonies were measured using Image J software.

**Immunofluorescent staining**

The monoculture and co-culture constructs were fixed with 4% formaldehyde/PBS and were either stored at 4°C or processed for cryosection. Constructs for cryosection were prepared by immersing them in OCT Tissue Teck/PBS (1:1) solution for 45 min followed by another 45 min in
OCT Tissue Tek. Then, the hydrogels were loaded into cryomoulds, fully immersed in OCT Tissue Tek before freezing them with liquid nitrogen. Hydrogels were stored at -80 °C until ready for sectioning. 10μm thick sections were permeabilised with 0.2% Triton-X for 10min and blocked for 1 hr with 1% BSA solution before incubation in primary and fluorescently conjugated secondary antibodies. Samples were counterstained with Phallodin and DAPI for 40 min. Images were captured using Leica SP5 CLSM (Leica).

**RNA isolation and quantitative Real-Time PCR (qRT-PCR)**

RNA extraction was performed with Trizol (Invitrogen) according to the manufacturer's instruction. For 3D cultures, three hydrogels were pooled into an eppendorf tube before performing RNA extractions with the Trizol reagent. RNA concentration was quantified using the Nanodrop-1000 (ND-1000). Samples with a 260/280 ratio higher than 1.7 were used for subsequent procedures. The samples were then treated with DNase Amp grade I and reverse-transcribed using the cDNA synthesis for qPCR kit (Invitrogen). The qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and data was analysed with SDS2.3 software as described previously [131]. The sequences of all primers used are as listed in Table 5.1.

<table>
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<th>Forward</th>
<th>Reverse</th>
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<td>IL-6</td>
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</tr>
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Cell lysate preparation and Western blotting assays
Monoculture and co-cultures of treated and non treated groups were harvested for lysate preparation in lysis buffer (1% Triton x-100, 150 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA and 25 mM NaF) containing protease inhibitor cocktail (Roche). For 3D cultures, hydrogels were dispersed using a pipette to release the embedded LNCaP cells. 20 μg of protein was loaded onto 10% sodium dodecyle sulphate (SDS) polyacrylamide gel and separated by electrophoresis for 2 hr at 120 V. Proteins were transferred to a nitrocellulose membrane via wet transfer for 1 hr at 100 V. After primary and secondary horseradish-peroxidase conjugated secondary antibody incubation, chemiluminescent Pierce ECL Western Blotting Substrate (Thermo Scientific) was added and membranes were exposed on X-ray films.

Statistical analysis
ANOVA post hoc LSD tests were used to test the statistical significance between hTEBC-LNCaP co-cultures and monoculture control groups unless specified. At least three independent in vitro experiments were conducted for all assays and analyses.

5.3 Results

Establishment of the 3D indirect co-culture model
The main criteria that was taken into consideration while developing this 3D co-culture model, is to emulate the CaP-bone microenvironment as closely as possible. This means a model that allows the LNCaP cells and hOBs, to interact in a paracrine manner, which reflects the process involved in CaP progression to bone metastasis. The co-culture model consists of LNCaP cells embedded in PEG hydrogels ‘glued’ to the hTEBC (Figure 5.1). From the cross section view of the construct, hOBs with fibroblastic morphology forming the layered mesh-like network is in close proximity with LNCaP colonies but not in direct physical contact (Figure 5.2A). LNCaP cells and hOBs conform to 3D structures and resemble morphologies of avascular tumors and woven bone, respectively. LNCaP cells in the PEG hydrogel form colonies of multicellular spherical mass. The hOBs on the other hand, form
Figure 5.1. A schematic illustration of the indirect 3D co-culture model set up. The model comprises of two constructs: (A) pre-prepared LNCaP cells embedded in PEG hydrogel discs and (B) mPCL-TCP scaffolds pre-seeded with hOBs and wrapped with hOB sheet, referred to as hTEBC. (C) Both constructs are then attached together by the PEG hydrogel to form the co-culture construct. (D) For each biological component to be analysed, the hydrogel and hTEBC can be separated for further sample preparation procedures. Scale bar: C= 2 mm.
Figure 5.2. Characterization of the 3D co-culture model and the effect of co-culturing LNCaP cells with hTEBC. (A) Morphology of LNCaP cells and hOBs in the 3D co-culture model. This model consists of 2 constructs: LNCaP/hydrogel and hTEBC that are assembled on top of one another, which is held by the PEG hydrogel mixture. 3D projections of CLSM images (20x, 1.0 NA) reveal Phalloidin/DAPI stainings of the actin filaments (red) and nuclei (blue) of LNCaP cells and hOBs. From the cross section view of the dissected construct (schematic, most left), hOBs form elongated and mesh-like morphology that surround the scaffold and attach to the scaffold struts while LNCaP cells appear as a multicellular mass.
LNCaP cells and hOBs are not in physical contact with each other as shown by the dotted line but are in close proximity, hence, forging a paracrine interaction. (B) The morphology and size of the LNCaP colonies after being co-cultured with hTEBC and LNCaP monocultures were examined for a 28 day period. The size of the colonies increases consistently from day 7 to day 28 in monocultures and co-cultures. The colony size of LNCaP cultures alone is larger when compared to co-cultures. (C) The size of LNCaP colonies in the monoculture and co-culture over a period of 28 days is shown as bars (median). The LNCaP colonies in the monoculture are significantly larger compared to the co-culture from day 14 onwards. Proliferation assay of LNCaP cells from monocultures and co-cultures from day 1 to day 28 reveals that the LNCaP cell growth (mean ± SE) is lower when co-cultured with hTEBC from day 14 onwards compared to LNCaP cells cultured in hydrogels alone. (D) Alkaline phosphatase (ALP) activities of hOBs (hTEBC) after co-culture with LNCaP cells for 28 day is compared to hTEBC cultures only. ALP activity (mean ± SE) of hOBs in co-cultures is significantly lower than the monoculture control. Significance of the comparisons is analysed with Student’s t-test and represented by (*) where p< 0.05. Scale bars: (A) 250 μm, (B) 100 μm.

layers of mesh-like networks that enclose and intertwine between the scaffold struts. Although the hydrogel and hTEBC are attached together, these two constructs can be easily separated. By co-culturing LNCaP cells and hOBs in this 3D model, we are able to examine the growth of LNCaP cells and perform other biochemical analysis in relation to their paracrine interactions.

Proliferation of LNCaP cells and alkaline phosphatase (ALP) activity in co-culture
To examine the influence of hTEBC on LNCaP cells, 3D projected CLSM images of the LNCaP colonies were analysed (Figure 5.2B). In PEG hydrogels, LNCaP colonies arising from single cells continue to increase in cell number throughout the 28 days culture period. Interestingly, morphological examination of the images show that the colony size is smaller when co-cultured with hTEBC. The colony size in monocultures increases dramatically after day 14 and remain constant at day 21 and day 28. The LNCaP colonies in the co-culture however, only increase in size from day 7 to 14 but do not increase in size thereafter. When the colony size in co-cultures is compared to monocultures, more than three fold decrease in size is detected in both day 21 and 28 of co-cultures (Figure 5.2C, left). This observation has lead to the quantification of cell growth. The growth of LNCaP colonies measured by the DNA content also yield a similar trend to the colony size when co-cultured with the hTEBC (Figure 5.2C, right). The smaller colony size in the co-culture is reflected in the proliferation assay as it
shows significantly lower growth of LNCaP cells when co-cultured with hTEBC as compared to monocultures after 7 days of culture. While the growth of LNCaP monocultures reaches a plateau after day 21, LNCaP cells in co-culture continue to grow linearly up to day 28. At day 28, growth in the co-culture is about 1.2 fold less than the monoculture. In order to test if the bone secreted factors are affecting the LNCaP cell growth, we have also measured proliferation of LNCaP cells cultured in hOB conditioned media (CM). Similarly, we found suppressed proliferation when LNCaP cells were cultured in CM compared to the normal growth media (Supplementary Figure 5.1).

Next, evaluation of the ALP activity of hOBs was performed to detect changes to the hOB activity when co-cultured with LNCaP cells (Figure 2D). The absorbance value represents the ALP activity measured from one hTEBC. By co-culturing the LNCaP cells and hOBs, not only is the cell proliferation of LNCaP cells affected, ALP activity of hOBs is also altered. As shown by the absorbance level normalized to total protein, ALP activity of hOB from day 28 co-cultures are significantly lower compared to the hOB control from hTEBC alone but higher than LNCaP monocultures. Since the protein level of hTEBC from both culture conditions is similar (data not shown), this indicates that the reduced production of ALP seen here is not due to the reduction of total intracellular proteins.

The effects of R1881 on gene expression profile of LNCaP/hTEBC co-culture
In order to apply this 3D co-culture model for studying the influence of androgens on cell CaP cells and hOBs, the monoculture and co-culture were treated with R1881 (1 nM) for 48 hours before cells were subjected to qRT-PCR analysis. The expression of androgen receptor (AR) and its target genes after R1881 stimulation was investigated (Figure 5.3A). As anticipated, these genes such as KLK2, KLK3/PSA and KLK4 are all elevated upon treatment with R1881 in both LNCaP cells of monocultures and co-cultures when compared to non treated control groups. Surprisingly, even in androgen free co-cultures the expression of KLK2, PSA and KLK4 is higher than non treated LNCaP monocultures particularly for KLK4 (p<0.01). Low KLK
Figure 5.3. Gene expression of LNCaP cells and hOBs in monocultures and cocultures in response to R1881. Data is graphed as mean ± SE. These genes encode for (A) target genes of the AR signalling pathway, (B) enzymes involved in lipid and androgen production, (C) EMT markers, (D) growth factors and (E) bone markers and chemokines. The increase in expression of KLK2, PSA and KLK4 are apparent in treated LNCaP cells. All KLKs show upregulation in untreated LNCaP cells from co-cultures compared to untreated LNCaP monocultures. No significant changes are detected in AR expression. FASN is upregulated upon treatment in LNCaP monoculture and co-cultures while RDH5 is not affected by the treatment. However, more RDH5 transcripts are detected in hTEBC monocultures compared to hTEBC co-cultures in treated and non treated conditions. Vimentin expression decreases in hTEBC co-cultures compared to monocultures while no changes are detected in LNCaP cells. In the presence of R1881, VEGF is upregulated in LNCaP cells co-cultured with hTEBC compared to monocultures. Some of the bone related markers (TGFβ1 and OCN) are downregulated in hTEBC upon co-culture with LNCaP cells. Bone matrix protein, OCN and transcription factor RUNX2 are specifically expressed by hOBs. IL-6 is also more expressed in hOB compared to LNCaP cells. Bars represent groups of: LNCaP monoculture ( ), LNCaP from co-culture ( ), hTEBC monoculture ( ) and hTEBC from co-culture ( ). (*) denotes significant difference (p<0.05) between treated and non treated LNCaP cells or hOBs within the same group either from monocultures or co-cultures. (Δ) denotes significant difference (p<0.05) between monocultures and co-cultures of LNCaP cells or hOBs receiving the same treatment. Triplicate samples were analysed from three independent experiments.
expression is also detected in hOB isolated from the R1881 treated co-culture but not in the hTEBC culture alone. It is suspected that a small population of LNCaP cells may have contaminated the hTEBC when the two constructs were being separated. Upregulation of these KLK genes upon R1881 treatment indicates that AR signalling is activated, however, this has no effect on the transcriptional level of AR. Other genes that are important in steroidogenesis and lipidogenesis of LNCaP cells were also evaluated. RDH5 and FASN are genes responsible for synthesis of dihydrotestosterone and fatty acid respectively (Figure 5.3B). In the LNCaP cells, no significant change to RDH5 was detected regardless of treatment condition. Surprisingly, RDH5 is also expressed by the hOB, in fact, at a higher level compared to the LNCaP cells. In contrast to RDH5, FASN is upregulated in LNCaP cells upon R1881 treatment. However, FASN is not expressed by hOBs in hTEBC monocultures but is slightly elevated in the treated co-cultures.

In cancer progression, EMT is often associated with cancer cells acquiring an invasive behaviour. This is characterised by the increased expression of mesenchymal markers and decreased expression of epithelial markers [224]. The CDH1 (E-cadherin) expressed by LNCaP cells does not change in expression when co-cultured with hTEBCs and is not affected by the R1881 treatment (Figure 5.3C). Similarly, another epithelial marker, the luminal epithelial marker CK8 is highly expressed by LNCaP cells compared to hOBs, especially when treated with R1881. The upregulation of CK8 is apparent in both treated LNCaP monocultures and co-cultures. Expression of Vimentin is higher in hOBs compared to LNCaP cells, which confirms the mesenchymal phenotype of the hOBs. In the co-culture however, Vimentin expression is downregulated.

Growth factors have been implicated in CaP cells homing to the bone site as well as development of bone metastasis. This study shows that the essential growth factors involved in bone metastasis such as VEGF and TGFβ1 are expressed by LNCaP cells and hOBs (Figure. 5.3D). Expression of VEGF is highly upregulated in LNCaP cells from monocultures and co-cultures after treatment with R1881. This suggests that VEGF is also regulated by the AR activity. VEGF is also upregulated in treated LNCaP
cells after being co-cultured with hTEBC compared to monocultures. TGFβ1 is highly expressed by hOBs but low in LNCaP cells. Higher expression of TGFβ1 in hOBs is consistent with the finding that this growth factor is produced abundantly by bone cells and stored in the bone matrix. Regardless of the presence of R1881, the TGFβ1 expression of hOBs from monocultures is higher than the co-cultures.

One other factor that could favour the colonization of CaP cells to the bone site besides bone-derived factors is the bone ECM proteins. The bone matrix is rich in proteins such as osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP) and collagen, which have been hypothesised to provide favourable conditions for the growth of CaP cells [39, 225, 226]. Interestingly, CaP cells too were reported to express some bone markers and the osteogenic transcription factor, RUNX2. This osteomimicry of CaP cells to bone is proposed to promote survival of CaP cells in the bone stroma [227]. Therefore, the expression of OCN and RUNX2 were investigated here (Figure. 5.3E). Our results reveal that OCN is expressed by hOBs but not LNCaP cells. OCN is downregulated in hOB co-cultured with LNCaP cells compared to hTEBC monocultures. A similar trend is also observed for RUNX2 expression in hOBs. However, the expression of RUNX2 in LNCaP cells remains low compared to hOBs. Interleukins are also known to play a role in inducing bone lesions particularly in the osteolytic process. Here, it is shown that IL-6 expression is higher in hOBs than LNCaP cells (Figure. 5.3E).

The effects of R1881 on protein production in LNCaP/hTEBC co-culture

To further quantify the levels of intracellular proteins in LNCaP cells and hOBs after R1881 stimulation, Western blotting was performed on selected markers that correspond to the qRT-PCR data (Figure 5.4). Analysis of the immunoblots reveals that PSA is increased in LNCaP cells when both monocultures and co-cultures were treated with R1881 in comparison to untreated monocultures. Interestingly, the PSA level of LNCaP cells co-cultured with hTEBC under the absence of R1881 is comparable to the treated co-cultures. PSA synthesis is significantly higher in LNCaP cells from co-cultures compared to corresponding monocultures of both untreated and
Figure 5.4. Western blot analysis of LNCaP cells and hOBs lysates in monocultures and co-cultures in response to R1881. Proteins were quantified from immunoblots and presented as mean ± SE in bar charts. An increase in proteins such as PSA, FASN, VEGF and CK8 with R1881 stimulation is detected in LNCaP cells from both monocultures and co-cultures. There is no significant change in AR production within the LNCaP group despite administration of R1881. The PSA level in untreated/treated LNCaP cells co-cultured with hTEBC is higher than untreated/treated LNCaP cultures only (check significance). Luminal epithelial marker, CK8, is expressed only in LNCaP cells while Vimentin is expressed by
hOBs only, confirming the mesenchymal phenotype of hOBs. However, Vimentin decreases in the co-cultures. A representative immunoblot (bottom right) shows the differential levels of protein of interest from the monocultures and co-cultures with and without R1881 treatment.

Bars represent groups of: LNCaP monoculture ( ), LNCaP from co-culture ( ), hTEBC monoculture ( ) and hTEBC from co-culture ( ). (*) denotes significant difference (p<0.05) between treated and non treated samples within the same group either from monocultures or co-cultures. (Δ) denotes significant difference (p<0.05) between monoculture and co-cultures receiving the same treatment. Analysis represents three independent experiments. Duplicate samples were analysed from three independent experiments.

treated condition. In addition, the hOBs from co-cultures also express more PSA than the hOB monocultures irrespective of R1881 treatment. This may suggest possible contamination of LNCaP cells in the hTEBC that had been separated. However, the expression of epithelial markers at mRNA and protein levels in hOB from co-cultures indicates otherwise. These markers like E-cadherin and CK8 are not significantly elevated upon co-culture. Moreover, the AR level of hOBs from co-cultures is also similar to the monocultures regardless of R1881 treatment.

Changes in PSA and AR protein production are consistent with their gene expression. Like the PSA, FASN is also elevated in LNCaP cells upon treatment in both monocultures and co-cultures when compared to the non treated LNCaP control groups. Both markers are positively regulated at mRNA and protein levels by R1881 treatment in LNCaP cells but not in hOBs. CK8, which is only produced by the LNCaP cells, is significantly elevated with R1881 treatment in LNCaP monocultures and co-cultures as compared to the non treated groups. Vimentin on the other hand is more exclusively produced by the hOBs due to their mesenchymal phenotype decreases in the co-culture, which is consistent with the change at transcription level.
5.4 Discussion

Culturing prostate cancer cells \textit{ex vivo} that differentiate and maintain \textit{in vivo} characteristics holds great promise not only for the pragmatic revelations of cell function but also in tissue engineering and regenerative medicine. Lack of a \textit{de novo} ECM milieu, which plays a crucial role in emission of physical and chemical signals besides providing structural support to cells, is a major setback in current \textit{in vitro} cancer cell culture. Hence, to translate the outcome of cancer research for clinical applications, it is important that cell culture based models should incorporate both the 3D organization and multi cellular complexity of a tissue while allowing experimental interventions in a desirable manner. To date, such models are rare and still in the developmental stage. Herein, we have established an indirect 3D \textit{in vitro} co-culture model to study the paracrine interaction between CaP cells and hOBs, a vital relationship that underlies the bone metastasis process. This 3D model consists of hydrogel-encapsulated LNCaP cells and hTEBC that allows separation of both cell types for analyses of each biological component. Moreover, experimental parameters can be easily modified and the culture condition is well controlled. Very importantly, LNCaP cells and hOBs are able to interact with their surrounding matrix and form 3D structures within these matrices.

These are the features that distinguish our 3D co-culture model from other current models. In the PEG hydrogel, LNCaP cells form a multicellular mass that resembles avascular tumor. Multiples studies in the past have proven that these aggregates mimic the behaviour of cells \textit{in vivo} [71-73]. Hence, the hydrogel encapsulated LNCaP cells are integrated into this more complex 3D co-culture model. From the examination of these co-cultures, the LNCaP cells and hOBs not only bear morphological resemblance to native tissues but they also retain the expression and production of LNCAP markers (E-cadherin, CK8, PSA, AR) and bone markers (Vimentin, OCN, RUNX2). In this study, we have demonstrated that the paracrine interaction between LNCaP cells and hOBs influences cell growth, gene expression and protein synthesis.

When LNCaP cells were co-cultured with hTEBCs, a decrease in colony size and proliferation of LNCaP cells was observed when compared to
LNCaP cultures alone. These findings contradict previous in vitro studies that reported enhancement of CaP cell proliferation when co-cultured with a human bone stromal cell line or after culturing LNCaP cells with conditioned media of hOBs in monolayer anchorage-dependent cultures [110, 228]. This inconsistency could be attributed to the geometry of the culture condition itself (2D vs 3D) or other variation in culture media preparation. The growth of LNCaP cells in this 3D co-culture system is likely more similar to the anchorage-independent growth. It has been shown that the androgen independent prostate cancer bone metastasis-derived sublines were more slow growing compared to parental cell C4-2 on agar [229]. Taken together this suggests that the the bone environment or bone derived factors do not necessarily increase CaP cell growth. Nutrient limitations are ruled out as the contributing factor because our previous data confirmed that molecules with the size of essential growth factors such as VEGF, TGFβ and PDGF can diffuse into the hydrogel. Moreover, the hTEBC is a highly porous construct and culture media was constantly replenished. Therefore, a lack of nutrients is unlikely the reason for the suppressed LNCaP cell growth in the co-culture. Instead, a combination of factors secreted by LNCaP cells and hOBs may effectively have a negative effect on the LNCaP cell growth.

Although the bone microenvironment is conducive to CaP growth as shown in clinical samples and in vivo studies, CaP cells homing to the bone site do not always develop into metastasis. Cancer cells are known to remain dormant for years before they develop into tumors. Therefore, it is not surprising that LNCaP cells from the co-culture do not grow as rapidly as the LNCaP monocultures. However, more studies are required to identify the soluble factors that could be suppressing the growth. In the co-culture, hOBs are also affected where a decline in the ALP activity and expression of bone markers, OCN and TGFβ1 are observed, indicating a decrease in hOB proliferation and differentiation. A decrease in osteoblast activity or bone turnover is not unusual as shown by Krishnan et al. (2010) [121] They also found a decline in production of OCN (mineralization marker) when metastatic breast cancer cells were co-cultured with osteoblastic tissues [121]. Take together, we propose that the CaP/hOB paracrine interaction does not completely hinder the growth of LNCaP cells but may have
suppressed the osteoblastic activity, a condition that reflects the early stage of the bone metastasis process [29, 37, 45]. Other molecular and protein changes (Vimentin, PSA) to hOBs from hTEBC co-cultures compared to monocultures suggest that the phenotype of hOBs is also altered, acquiring a less osteoblast-like feature. In fact, by producing PSA this suggests that the hOBs are behaving more like the LNCaP cells, which respond to androgens.

We have also utilised this model for studying the response of cells to the synthetic androgen, R1881, to further simulate the clinical situation in the presence of androgens. From the gene and protein expression studies, upregulation of AR target genes, KLK2, PSA, and KLK4 proves that the AR signalling pathway is activated upon R1881 treatment. Interestingly, even without R1881, LNCaP cells co-cultured with hTEBCs are expressing PSA protein at a similar level to treated co-cultures. This suggests that bone derived factors may have induced PSA production in LNCaP cells in the absence of R1881 treatment. Others have reported that the bone derived factor, IL-6, was found at sufficient level in conditioned media of osteoblast or bone-like cell cultures to upregulate PSA expression and increase secretion of PSA [110, 230]. IL-6, which is elevated in serum of castrate resistant CaP patients is able to activate AR, independent of androgen binding via STAT3 and MAPK pathways [230-232]. When hTEBC from this study was analysed, IL-6 transcripts were detected in hOBs. Our observations may explain why castrate resistant CaP cells can survive in the bone and continue to produce PSA. Collectively, our findings also agree that PSA is elevated in hOBs when hTEBCs are co-cultured with LNCaP cells as shown in qRT-PCR and Western blot assays.

Besides PSA, other proteins associated with CaP progression and bone metastasis such as FASN and RDH5 [206, 233, 234] are also detected at the mRNA or/and protein levels in the LNCAP cells or hOBs. FASN is essential for lipidogenesis and is found to be upregulated by R1881 treatment in both monoculture and co-cultures. Unexpectedly, the mRNA level of RDH5, an enzyme vital for the DHT synthesis in CaP cells is found more abundant in hOBs compared to LNCaP cells. It is possible that RDH5 from hOBs also facilitate in androgen production of CaP cells. De novo synthesis of DHT by the CaP cells can increase serum PSA, which is often
observed in of castrate resistant CaP patients [235]. This could possibly abrogate the dependency of CaP cells on systemic androgens. Hence, this also rationalizes the ability of CaP cells to cause bone metastasis even after androgen deprivation therapy.

Here, we also queried whether the CaP/hOB paracrine interaction influences the invasive behaviour of LNCAP cells by examining the E-cad, CK8 and vimentin markers. These EMT markers are unchanged upon co-culture. It is not sufficient to screen for these markers only as there are other EMT signature markers that could implicate a change in cell phenotype. As reported by Sethi et al. [236], there were no significant difference in E-cadherin and Vimentin protein expression between primary CaP and bone metastasis. However, another EMT marker, NOTCH-1, was expressed at a higher level in bone metastasis compared to primary CaP [236]. Screening other factors such as N-cadherin, Fibronectin, TWIST, ZEB1, SNAIL and SLUG would provide a more conclusive validation of EMT changes. The androgen regulated VEGF is highly expressed in LNCaP cells while transcription of TGFβ1 is more abundant in hOBs. These GFs affect the growth and survival of both cell types, which essentially influence the cell homeostasis in the co-culture. Presence of GFs also regulates expression and secretion of other factors that could in turn influence cell behaviour.

From this established indirect 3D in vitro co-culture model, we can conclude that by using this dual construct approach, it allows culturing of LNCaP cells and hOBs within a distinct 3D microenvironment. Having two different cellular components that produce different soluble factors clearly demonstrate a paracrine interaction of both cell types. This has triggered several cellular and molecular responses that reflect the in vivo CaP-bone relationship. We have proven that this in vitro 3D co-culture model can be used as a tool for more in depth investigations on CaP bone metastasis mechanisms. Basic features of this model can also be modified to better address other specific biological questions and will be a valuable in vitro tool for anticancer drug screening.
Chapter 6: Conclusions
Chapter 6: Conclusions

In cancer pathogenesis, the interplay between cancer cells and their microenvironment influences cell tumorigenicity and plays an important role in cancer progression. The cell microenvironment is undoubtedly crucial for modulation of cell behaviour in terms of growth, differentiation and migration. Therefore, in order to correctly interpret results from *in vitro* studies and extrapolate to the clinical picture, a more physiological *in vitro* system that current 2D cultures lack is much needed. This leads to the development of 3D *in vitro* models that encompass the microenvironment elements of native tissues. In addition, 3D cultures also offer valuable estimations of drug efficacy prior to testing in *vivo* models. Hence, this PhD project aimed to establish a 3D *in vitro* model for studying the different stages of cancer progression.

With the aid of TE technology platforms, it is now possible to culture cancer cells in 3D for the purpose of testing cancer-based hypotheses and mechanisms. For this PhD project, biomaterials and techniques used in the TE field were adopted for culturing CaP cells. Two 3D constructs were developed for this PhD project which are: 1) the androgen dependent CaP cell line, LNCaP cells, embedded within synthetic PEG-based hydrogels and 2) hTEBC fabricated from mPCL-TCP scaffold overgrown with primary hOBs. These constructs have been used successfully to test different hypotheses. From these 3D culture studies, some conclusions have been drawn and are summarised below.

The first part of the study aimed to establish a 3D model for culturing LNCaP cells, and to characterise these cells cultured within the PEG hydrogel in comparison to cells in monolayer cultures. From this study, the synthetic PEG hydrogel was selected as the matrix of choice due to the flexibility of tuning the stiffness of the hydrogel to approximate the mechanical properties of the early stage of prostate tumor formation and the flexibility of being able to modify the biochemical properties. Clearly, the behaviour, such as the growth profile of prostate cancer cells, like LNCaP cells, is dependent on the mechanical property of the hydrogel. The ability of soluble factors (e.g. growth factors, serum proteins etc. and nutrients from
the culture media to fully penetrate the hydrogel to provide a conducive environment for cell growth is an important aspect from a biomimetic point of view. The studies performed in chapter 3 show that LNCaP cells can proliferate from single cells to form multicellular spheroids, which confer the monoclonal entity of each cell colony. The characteristics of the growing LNCaP colonies resemble the avascular tumor not just in terms of morphology but also in their growth kinetics. Given the more physiological features of slower growing LNCaP cells in the hydrogel, these cultures can be studied for a period of up to 28 days. In contrast, 2D cultures are performed for only up to 4 days. It was concluded from our studies that these 3D cell culture features reflect an early developmental stage of CaP, which also undergo a slow cell division from one parent cell to form primary prostate tumors.

As revealed by the morphological examination of LNCaP colonies in the hydrogel cultures, the densely packed LNCaP cells with prominent cell-cell contact could influence the accessibility of nutrients and oxygen, hence the cellular response to R1881 in the 3D culture differs significantly when compared to the 2D culture. As colony size increases at the later stage of the culture, cells towards the middle of the colony may experience a greater nutrient/oxygen deprivation which could lead to cell apoptosis and hypoxia. A combination of difference in mass transfer, intracellular communication and cell-matrix interaction in the hydrogel and in 2D cultures may have influenced the lack of AR translocation. Further investigation would be required to unravel this unexpected phenomenon. This in turn has also led to the varied cell response to R1881 and differential expressions of genes involved in lipid/steroid metabolism in 3D cultures compared to 2D cultures in the absence of R1881.

Despite the ability to mimic many aspects of the tumour microenvironment and tumor physiology, this 3D model presents technical challenges, too. Besides physical preparations of hydrogels that require trained operators, the extraction of RNA and protein from the cells is also more difficult compared to extraction from 2D cultures. The efficiency of the RNA and protein extraction from the PEG-based matrices are compromised as the hydrogel is not easily degraded by the lysis buffers/reagents, hence
there is a high loss of samples, which inconveniently means more starting material is required. In addition, imaging 3D hydrogels depends on the fluorescent signals of immunostained cells. The penetration of these signals through the non-transparent hydrogel is weakened; hence the signal detection of the hydrogel is compromised. This in return produces an image that may not project a distinct localisation of protein of interests.

Like all cancers, carcinogenesis of CaP involves multiple stages. To develop a 3D model that only recapitulates one stage of the disease will not satisfy the current cancer research needs. So far, 3D models for the more advanced stage of cancer progression, bone metastasis, are only beginning to emerge. These models still require further improvement on mimicking the bone microenvironment. In order to simulate the CaP-bone interaction in a 3D model, a direct co-culture model was developed in the second part of the PhD thesis (chapter 4), which aimed to investigate the interactions between the CaP cells and hOBs. The hTEBC used for this study is highly reproducible and robust. Most importantly, it can be fabricated from primary hOBs, and maintain hOB viability up to 80-90%; without any significant differences between patients from which the hOBs were isolated from. This high cell viability is vital to enforce a communication between hOBs and CaP cells when they are co-cultured together. Using both cell types of the same species for this study confers this model an all human 3D model, which is rare in current 3D bone metastasis models. The hTEBC allows CaP cells to attach and grow on the hOB matrix. The interaction between CaP cells and hOBs are apparent when these cells are in physical contact with each other. As CaP cells, PC3 and LNCaP represent different phenotypes of the CaP cell line; this is reflected in their interaction with hOBs in terms of their morphology and production of MMP9. In the direct co-culture, the remodelling of the hOB matrix is not observed, however, the formation of aggregates on the matrix by LNCaP cells and production of MMP9 when PC3 cells were co-cultured with the hTEBC is in agreement with the osteoblastic and osteolytic nature of each CaP cell line.

To further test the application of this 3D co-culture model by simulating the clinical situation, under the presence of the synthetic androgen R1881, molecular changes of androgen-sensitive LNCaP cells and hOBs in response
to R1881 was investigated. This is a proof of principle that the model can be used for testing compounds that may exert certain effects on these cells under a CaP-hOB microenvironment. Although this co-culture model very well recapitulates the CaP-hOB interaction, large standard deviations of biological responses was often seen. This could be attributed to the inconsistent seeding efficiency of the CaP cells on the hTEBC as the CaP cells are not spatially confined within a matrix. Hence, variation of the CaP cells-hOBs ratio could potentially yield different cell response between biological samples. Analysing individual biological component of this co-culture model is almost not possible because it is difficult to segregate both cell types from the co-cultures for downstream biochemical analyses and gene expression profiling. To achieve statistical significance for these analyses and improve separation of cell types, a large sample size is required. Even though fluorescent labelled cells can be sorted by Fluorescence-activated cell sorting (FACS), a complete segregation of the CaP cells and hOBs is not guaranteed. To address this issue, using models that do not involve mixing of the CaP cells and hOBs is a potential approach to allow analysis of gene and protein expression profiles of each cell type.

From a direct co-culture model, an indirect CaP cell-hOB co-culture model consisting of hydrogels embedded with LNCaP cells and hTEBCs was developed in the third part of the PhD thesis. Despite the absence of physical cell-cell contact between the two cell types this co-culture model is an attractive tool to investigate the paracrine interaction of LNCaP cells and hOBs. The cells cultured in this system are viable and can proliferate in this microenvironment. In this study, our results are consistent with cellular responses seen in the early stage of bone metastasis when CaP cells start to colonise the bone stroma, and where bone resorption outweighs the osteoblast activity. Suppression of the ALP activity of hOBs is an indication that osteoblastic reactions are not favoured during this initial phase. Using this model again to study the effects of R1881 on the cells, it is evident that the gene expression levels are consistent with the protein production. The extensive gene expression profiling confirms that the LNCaP cells are responsive to R1881 treatment and that both cell types still retain their
respective identity in such a 3D model by expressing their distinct markers (Ecad, CK8, vimentin, OCN).

Another biological response that surfaced from the paracrine interaction involves upregulation of the KLKs without stimulation of R1881. This suggests that hOB derived factors are able to activate the AR, bypassing binding of androgens to AR. One of these factors is IL-6 that has already been proven to transactivate the AR and found to be expressed in hOBs in this study. Therefore, in this study it is possible that IL-6 is responsible for triggering non-androgenic expression of KLK genes in LNCaP cells in the absence of R1881. In conjunction with that, it was also found that RDH5 is produced by the hOB, which suggests that hOBs could facilitate in androgen production of LNCaP cells. Taken together, this response reflects the physiology of castrate resistant CaP where KLK3/PSA is elevated when androgen deprivation therapy fails.

As the LNCaP cells are co-cultured with the hTEBC, they too undergo a similar growth pattern and morphogenesis as the LNCaP cells in monoculture. The issue with inconsistent cell seeding is overcome as the LNCaP cells are embedded within the hydrogel. Therefore, the number of cells co-cultured with the hTEBC is well controlled. In contrary to the direct co-culture model, the hydrogel and hTEBC can be physically separated to be processed for analyses with minimal interference from the other cell type. However, the carryover of LNCaP cells to the hTEBC is unavoidable as shown from the gene expression data. The use of this indirect co-culture model unfortunately does not preclude the difficulty in extracting sufficient RNA and protein for qRT-PCR and western blotting. Hence, the experimental protocol requires a larger sample size or bigger constructs.

In conclusion, we have shown that tissue engineering by using 3D models provides a highly useful system for studying cancer biology that includes all stages of cancer development and progression. The versatility of 3D models truly has offered to cancer research great opportunities to address multiple biological questions in a more physiological manner. Not only will this enhance our basic understanding of cancer biology but also delineating the more complex mechanisms of CaP pathogenesis. This will
ultimately benefit the development of anticancer therapy and improve disease management.

6.1 Recommendations for future work

3D in vitro cancer studies have progressed tremendously over the last decade, assisted by the advances of biomaterials and procedures used for 3D cultures. The evolution of the matrix in 3D models, one of the key elements of cell microenvironment, has allowed diversity in experimental strategies. As shown in this study, using the synthetic PEG-based hydrogel opens up avenues for multiple mechanistic studies in particular, those related to tumorigenesis. With this system, the role of extrinsic factors and interaction with stroma cells in tumorigenesis (e.g. biomolecule and biophysical components), which would be difficult to control in in vivo models can be independently examined. For example, further understanding of angiogenesis in tumor development can also be gained by allowing the cancer cells to interact with either the angiogenic factor itself (VEGF) or the endothelial cells present in the hydrogel. Besides using this model as monocultures, the hydrogels can be adapted for co-culturing with other stromal cells (e.g. endothelial cells, fibroblasts) using or in combination of appropriate scaffolds/matrices to further approximate the tumor microenvironment. While it is a great tool for studying cancer development and progression and potentially a tool for testing anticancer drugs, a thorough characterization of the 3D model is required to validate the feasibility and suitability of the model.

Following the first study (chapter 3), further exploration in terms of molecular changes should be performed. From the microarray gene expression profiling, identification of key pathways or factors that contribute to the elevated expression of androgen regulated genes when not stimulated by androgen in 3D cultures compared to 2D culture (decreased dependence on androgen in 3D cultures) could be an important breakthrough. This could possibly offer insights into the underlying cause of hormone dependent CaP progressing to castrate resistant CaP, a condition that severely threatens any chance of survival in CaP patients. Additionally, a phenotypic
characterization of the androgen independent and bone metastatic LNCaP sublines (C4, C4-2B) cultured in the PEG hydrogels would also present a relevant comparison to the parental cell line with regards to hormone dependency and metastatic potential. Potentially, this work could also be extended to study and compare other CaP cell lines that induce different bone metastatic lesions (e.g. using the osteolytic cell line, PC3 vs osteoblastic cell line, MDA PCa 2B). This will greatly enrich the existing in vitro profile of these cell lines that can be linked to the clinical situations. However, due to genetic manipulation of these transformed cell lines, they may not fully represent the native phenotype of CaP cells, which could limit the interpretation of results. Therefore, instead of relying on transformed cell lines, these established 3D models can be used for culturing primary cells isolated from clinical specimens. These cells that better represent the disease state may be a better option for profiling the disease of various Gleason grade, hormone dependency and metastatic potential. This can then supplement the current knowledge of the various mechanisms involved in development of clinically relevant CaP phenotypes.

Since the PEG hydrogel is only beginning to be utilized for culturing cancer cells, apart from characterization of LNCaP cells grown in the PEG hydrogel, a comparison between the cell behaviour cultured in PEG hydrogel and the two other commonly used matrices, Matrigel™ and collagen type I would be essential in providing important information on the morphogenesis, cell differentiation and cell signalling pathways and the differences between cells in 3D matrices. This information can facilitate in the selection of the matrices to culture CaP cells to suit experimental purposes. One other area that can be explored is the matrix itself. Instead of functionalizing PEG with the fibronectin-integrin binding sequence (RGD), it can be conjugated with laminin (YIGSR) or other ECM motifs. Since laminin is the major component of the basement membrane, which encapsulates the prostate gland, it would be relevant to evaluate the effect this ligand-receptor interaction has on the cell phenotype. This would also test the hypothesis that this matrix can better mimic the biochemical property of the native ECM surrounding glandular tissues and could induce cell polarity via the cell-matrix interaction. So far, culturing CaP cells in a PEG hydrogel functionalised with YIGSR has not
been done. This may provide another option for studying cell polarity, acini formation without being too reliant on Matrigel™.

It is evident that the LNCaP cells and hOBs can affect each other directly and indirectly. Our reliable and feasible 3D co-culture model could be used for characterizing metastatic CaP cells from patients. These bone metastasis CaP cells that are often heterogenous may elicit different cellular and molecular response in the bone environment. Hence, CaP cell aggression and the cellular changes during bone homeostasis may vary between patients, causing management of the disease to be far from straightforward. With this 3D co-culture model, it provides a means for capturing the physiological events occurring when the CaP cells encounter the bone (e.g. bone matrix remodelling, cellular and molecular changes to CaP cells and hOBs) in vitro that is specific for each patient. Thus, it provides information that could improve therapeutic strategies.

The 3D co-culture model developed in this study, using only two cell types (LNCaP and hOB cells) is a starting point to improve mimicry of the bone microenvironment. Adaptation of the current 3D co-culture model to portray a more complex CaP cell/bone environment involving multiple cell types such as osteoclasts or bone marrow stroma cells could further improve the co-culture model. Following establishment and validation of this co-culture model, we can endeavour to engineer tumors/tumor stroma with in vivo-like cellular components by co-culturing non-cancerous cells like, cancer-associated fibroblasts, myofibroblasts, endothelial cells and inflammatory cells with cancerous cells. Since little is known about the interaction of CaP cells and their stroma (primary or secondary sites), a 3D heterotypic model may help to elucidate mechanisms involved in tumor development and progression of CaP. A physiological in vitro model that could specifically represent the clinical conditions with validation from human CaP xenografts would further enhance the strength of this 3D in vitro model. Using various analyses, in combination with genomic and proteomic studies, this has potential to improve our understanding of the mechanisms and factors that drive progression to advanced CaP (e.g castrate resistant and metastatic). Subsequently, heterotypic 3D culture is a challenge that would require collaborative participation of multidisciplinary experts, namely,
bioengineers, clinicians and cancer biologists to recreate a biomimetic model that is feasible and practical and which allows subsequent assays and examinations to be performed.

From the current direct co-culture study, additional analyses including examination of cell movement, morphology changes and growth of the CaP cells on the hOB matrix in real time can be carried out using live imaging. This will also provide additional information on how CaP cells can integrate into, or remodel the bone matrix, which can be linked to the phenotype of the CaP cells used. Interesting results found in the indirect co-culture study also require further investigative studies. In particular, the bone derived factors that play a role in the increased KLK expression in LNCaP cells under androgen deprived conditions may also be implicated in contributing to androgen independent CaP. Identification of these paracrine factors either at the genomic or proteomic levels may offer new therapeutic approaches to manage advanced CaP. As proven from the indirect co-culture model (chapter 5), it is now possible to segregate the CaP cells from hOBs. With this advantage, a more extensive analysis involving microarray gene expression should be considered. The molecular changes of these cells can further help to identify factors/targets or pathways that could be initiating or promoting metastasis.

Lastly, all of the 3D models presented so far have the potential to become a tool for drug development programs. These models can be further validated by comparison to in vivo models in terms of drug response and efficacy. The 3D in vitro model would be a supporting tool for in vivo studies that researchers may have no control over such as the biophysical component of the tumor microenvironment. Our 3D models are prospective tools for development of a more physiological in vitro system that could ultimately contribute to the existing understanding of cancer biology.
Supplementary materials

Chapter 3

Supplementary Figure 3.1. Stiffness of 2% PEG hydrogels after culture with LNCaP cells for 54 days. A small variation of the hydrogel’s stiffness was detected between the days of culture.

Supplementary Figure 3.2. 3D confocal image projections of 1.5-2.5% PEG hydrogels after 24 hr immersion in 1 mg/mL BSA-FITC solution. Images of the cut surface were taken to examine the penetration of the BSA-FITC (66kDa) across the thickness (1.5mm) of the hydrogel discs (green). It shows that BSA-FITC can still saturate the 2.5% PEG hydrogel. Dotted lines demarcate the border of the hydrogels. Scale bars, 100μm.
Supplementary Movie 3.1. Movie of LNCaP cells embedded in a 2% PEG hydrogel at day 7 of culture. The movie was taken with widefield microscopy from day 7 to day 12. This shows that spheroids are formed by cell division within the spheroid and not by aggregation of neighboring cells. No active cell migration was observed in the culture.

Supplementary Figure 3.3. CLSM 3D projection of the of LNCaP cells grown in Matrigel™ for 14 days. Cells embedded in Matrigel form spheroids with well defined shape similar to cells grown in PEG hydrogels. CLSM images are taken at 40x and 1.25 NA. Scale bar: 250 µm.
Supplementary Figure 3.4. 3D projection of a CLSM image of LNCaP cells grown in 2% PEG hydrogels for 28 days. Cultures were treated with R1881 for 48 hours before harvest. 10-14% of the colonies formed in the PEG hydrogel, show localisation of CK8 in the centre of the colony. CLSM images are taken at 40x and 1.25 NA. Scale bar: 75 µm.

Chapter 4

Supplementary Figure 4.1. A schematic outline of metabolism of cholesterol to dihydrotestosterone through the typical steroidogenic pathway and backdoor pathway (bold italics). Adapted from Locke et al Can Res (2008) [206].
Chapter 5

Supplementary Figure 5.1. Proliferation of LNCaP cells cultured in growth media and hOB conditioned media (OCM). LNCaP cells were seeded onto 24 well plates at cell density of $1 \times 10^4$/cm$^2$. Cells were cultured in normal RPMI growth media (+10% FBS) or OCM (+10% FBS) and harvested at day 1, 3, 5, 7 and 9. The fold change of total DNA compared to day 1 of harvested cells are plotted as mean ± SE. Growth of LNCaP cells in OCM is impeded after day 5 as compared to LNCaP cells grown in growth media.

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