Regulation of the Chemokine Receptors CXCR4, CXCR7, and the Androgen Receptor in Prostate Cancer

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**ABSTRACT**

The chemokine receptor CXCR4 contributes to tumour cell migration and invasion during the progression of prostate cancer. In particular, this pathway is central to the metastasis of prostate cancer to the bone marrow. Limited therapeutic options exist for prostate cancer patients who have progressed to advanced metastatic disease, and pharmacological interference of the chemokine network may serve to control tumour cell dissemination and the establishment of metastasis. A more detailed knowledge of the mechanisms regulating chemokine receptors is required, in order to further characterise and explore the capacity and effectiveness of targeting these pathways for therapeutic intervention in prostate cancer.

Here, the regulation of CXCR4 protein expression and function was investigated in relation to androgens and the extracellular matrix. Accumulating evidence of CXCR4 regulation by androgens and the androgen receptor have indicated that androgens not only promote the growth and development of prostate cancer, but may actively contribute to the metastatic progression of prostate through modulation of the chemokine network. In the current study, the endogenous protein expression and functionality of the androgen receptor were firstly characterised in the androgen-insensitive prostate cancer cell lines DU145 and PC3, using the androgen-sensitive LNCaP cells as a basis for comparison. Investigations were performed using two-dimensional culture in conjunction with the more physiologically relevant three-dimensional *in vitro* culture model. As expected, LNCaP cells expressed prostate-specific antigen and displayed androgen-sensitive growth regulation, indicative of a functional androgen receptor. The androgen-insensitive DU145 cell line remained androgen receptor-negative in both two-dimensional and three-dimensional culture conditions. Surprisingly, androgen receptor-negative PC3 cells displayed a clear induction of androgen receptor protein expression in three-dimensional culture. The growth of PC3 cells remained androgen-insensitive in three-dimensional culture, and although androgen receptor responded to treatment with androgens by undergoing nuclear translocation, no production of the androgen receptor-target gene, prostate-specific antigen, was detected. Furthermore, evidence of differential androgen receptor regulation by signalling pathway activity was observed between PC3 and LNCaP cells, revealing a divergence in androgen receptor regulation between androgen-sensitive LNCaP cells and androgen-insensitive PC3 cells.
Consistent with findings in the literature, androgen regulation of CXCR4 expression was demonstrated in LNCaP cells, although the functional consequences of this regulation were limited. Functional studies of ligand-induced signalling and LNCaP cell migration revealed that CXCR4 displayed limited functional responses in this cell line. The more invasive, androgen-insensitive DU145 and PC3 cell lines were found to express highly functional CXCR4, which mediated ligand-induced cell migration responses. The treatment of androgen receptor-positive three-dimensional PC3 cultures with androgens resulted in increased CXCR4 protein expression, similar to that observed in LNCaP; a response which was mediated by androgen receptor activity. However, the lack of prostate-specific antigen production in these PC3 cultures indicated limited androgen receptor transcriptional activity, despite nuclear translocation of the receptor in response to DHT. Further investigations indicated that androgen receptor signalling may contribute to CXCR4 regulation in PC3 cells, an effect mediated through differential pathways to that observed in LNCaP cells.

The alternative SDF-1α-binding receptor, CXCR7, has also been associated with prostate cancer progression via regulation of tumour growth and invasion. Studies of prostate cancer cell proliferation in two-dimensional culture revealed that CXCR7 was required to maintain the growth of LNCaP cells in depleted culturing conditions generated using charcoal-stripped FBS. Considering previous reports of a mutual regulation occurring between CXCR7 and CXCR4 in vitro, the regulation of these receptors were studied in three-dimensional culture. A marked up-regulation of both CXCR7 and CXCR4 protein was observed upon culturing PC3 cells in three dimensions. The expression of these proteins was found to co-localise at stellate projections, structures which penetrated into the surrounding matrix and were rich in matrix metalloproteinase protein expression. A crucial role for integrin β1 was demonstrated in the formation and maintenance of the PC3 stellate phenotype, as a mediator of cell-extracellular matrix interactions. Consistent with the close association between CXCR4 and CXCR7 protein expression with stellate projections, inhibition of integrin β1 resulted in reduced protein expression for both chemokine receptors. The results reported here indicated that the protein expression of CXCR7 and CXCR4 were linked with the more invasive, stellate phenotype of PC3 cells in three-dimensional culture in vitro. When considered in the context of chemokine receptors in the regulation of prostate cancer metastasis, these findings may have implications for inter-regulation between chemokine receptors, integrin β1, and the extracellular matrix.
potentially contributing to the progression of prostate cancer to the invasive, metastatic tumour cell phenotype characteristic of advanced disease.
STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. 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 in the dissertation itself.

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Debra L. Kiss
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LIST OF ABBREVIATIONS

2D = Two-dimensional
3D = Three-dimensional
ADT = Androgen deprivation therapy
AMACR = α-Methylacyl-coA racemase
ANOVA = Analysis of variance
APS = Ammonium persulphate
AR = Androgen receptor
ARE = Androgen responsive element
ATCC = American type culture collection
BCa = Breast cancer
Bcl-2 = B-cell lymphoma 2
BPH = Benign prostatic hyperplasia
BSA = Bovine serum albumin
Cat. No. = Catalogue Number
CCR1 = CC chemokine receptor 1
CCR5 = CC chemokine receptor 5
CCR6 = CC chemokine receptor 6
CCR7 = CC chemokine receptor 7
cDNA = Complementary DNA
CNS = Central nervous system
CRPC = Castration-resistant prostate cancer
CRS = Cell recovery solution
CS-FBS = Charcoal-stripped FBS
CT = Computed tomography
CTC = Circulating tumour cell
CTD = Carboxy-terminal domain
CXCR1 = CXC chemokine receptor 1
CXCR2 = CXC chemokine receptor 2
CXCR3 = CXC chemokine receptor 3
CXCR4 = CXC chemokine receptor 4
CXCR7 = CXC chemokine receptor 7
CYP17 = Cytochrome P450 17α-hydroxylase/17,20-lyase
DAPI = 4′,6-diamidino-2-phenylindole dihydrochloride
dNTP = Deoxyribonucleotide triphosphate
DBD = DNA-binding domain
DC = Dendritic cell
DHT = Di-hydroxytestosterone
DIC = Differential interference contrast microscopy
DNA = Deoxyribonucleic acid
DRE = Digital rectal exam
DS = Double stranded
ECM = Extracellular matrix
EGF = Epidermal growth factor
EHS = Engelbreth-holm-swarm (EHS)
ELISA = Enzyme-linked immunosorbent assay
EMT = Epithelial-to-mesenchymal transition
EPCA = Early PCa antigen
ERG = Avian V-ETS erythroblastosis virus E26 oncogene homolog
ERK 1/2 = Extracellular Signal-regulated kinase 1/2
ETS = E-twenty six
E.coli = Escherichia coli
FAK = Focal adhesion kinase
FBS = Fetal bovine serum
fPSA = Free prostate specific antigen
GAPDH = Glyceraldehyde 3-phosphate dehydrogenase
GCSF = Granulocyte colony stimulating factor
GEM = Genetically engineered mouse
GnRH = Gonadotropin-releasing hormone
GPCR = G protein-coupled receptor
HEK = Human embryonic kidney
Her2 = Human epidermal growth factor receptor 2
HIV = Human immunodeficiency virus
hK2 = Human kallikrein 2
HRP = Horseradish peroxidase
HSP = Heat shock protein
IGF = Insulin-like growth factor
IL-6 = Interleukin 6
IL-8 = Interleukin 8
ITAC = Interferon-inducible T cell alpha chemoattractant
JAK-STAT = Janus activated kinase-signal transducer and activator of transcription
KLF5 = Krüppel-like factor 5
LBD = Ligand binding domain
LH = Leutenising hormone
LHRH = Leutenising hormone releasing hormone
MAPK = Mitogen-activated protein kinase
mAR = Membrane androgen receptor
MIP-1α = Macrophage inflammatory protein-1 alpha
MMP = Matrix metalloproteinase
MMP-11 = Matrix metalloproteinase 11
MMP-9 = Matrix metalloproteinase 9
MRI = Magnetic resonance imaging
mTOR = Mammalian target of rapamycin
MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW = Molecular weight
N-Cadherin = Neural-cadherin
NCBI = National Centre for Biotechnology Information
NEB = New England Biolabs
NK = Natural killer
NTD = N (amino)-terminal domain
PAGE = Polyacrylamide gel electrophoresis
PBS = Phosphate-buffered saline
PCa = Prostate cancer
PCA3 = PCa antigen 3
PCR = Polymerase chain reaction
PFA = Paraformaldehyde
PGA = Polyglycolide
PI3K = Phosphoinositide 3-Kinase
PICP = C-Terminal pro-peptide of pro-collagen type 1
PIN = Prostatic intraepithelial neoplasia
PINP = N-Terminal pro-peptide of pro-collagen type 1
PLA = Polylactide
PLC = Phospholipase C
PLG/PLGA = Poly(lactide-co-glycolide)
PSA = Prostate specific antigen
PTEN = Phosphatase and tensin homolog
PVDF = Polyvinylidene fluoride
Rb = Retinoblastoma
RIPA = Radio-immunoprecipitation assay
RNA = Ribonucleic acid
ROI = Region of interest
RPMI = Roswell Park Memorial Institute
RT = Room temperature
RT-PCR = Reverse transcriptase polymerase chain reaction
SDF-1α = Stromal-derived factor-1 alpha
SDS = Sodium dodecyl sulphate
SDS-PAGE = Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S.E.M = Standard error of the mean
SFM = Serum-free culture medium
SIV = Simian immunodeficiency virus
TBS = Tris-buffered saline
TBST = Tris-buffered saline-tween 20
TC = Tissue culture
TEMED = Tetramethylethylenediamine
TGFβ = Transforming growth factor beta
TIMP = Tissue inhibitor of metalloproteinase
TIMP-2 = Tissue inhibitor of metalloproteinase 2
TMPRSS2 = Transmembrane protease, serine 2
TNM = Tumour, node and metastasis
TRAMP = Transgenic adenocarcinoma of the mouse prostate
uPA = Urokinase plasminogen activation axis
V = Volts
VEGF = Vascular endothelial growth factor
v/v = Volume per volume
w/v = Weight per volume
DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Debra L. Kiss
PUBLISHED WORKS BY THE AUTHOR INCORPORATED INTO THE THESIS

Included in this thesis are two published paper, the first of which has been incorporated into Chapters 3 and 4, and was co-authored with other researchers. My contribution to the co-authored paper was in undertaking Western blots and immunocytochemistry, and participating in scientific discussion relating to the manuscript.

The bibliographic details for these papers are as follows:-


Additionally, another manuscript has been published based on the results presented in Chapter 6. As first author, my contribution to this manuscript consisted of performing western blots, immunocytochemistry, image analysis, data interpretation and preparation of the manuscript. The details for this manuscript are as follows:-


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the published paper.

_________________________________
Debra L. Kiss

_________________________________
Professor Vicky M. Avery
ADDITIONAL MATERIAL RELEVANT TO THE THESIS BUT NOT FORMING PART OF IT

CONTRIBUTIONS OF OTHERS TO THE THESIS

Professor Vicky Avery has assisted with discussion and establishment of thesis aims, research hypotheses, choice of methods, critical analysis of experimental results, scientific discussions and in the structuring of the thesis. Professor Avery also performed editing and proofreading of the thesis itself.

Dr Greg Fechner assisted in structuring the aims of the research, project guidance, scientific discussion, and in editing and proofreading of the thesis.

Dr Louisa Windus provided assistance with project supervision, scientific discussion and in editing and proofreading of the thesis. Dr Windus also performed experimental work including immunocytochemistry, Western blots, image analysis and statistical analysis. The specific contributions of Dr Windus to the experimental results presented in this thesis were as follows:-

- Chapter 3: Figures 3.1, 3.3 - 3.8, and 3.10 - 3.12
- Chapter 4: Figures 4.1, 4.13 - 4.17, and 4.21 - 4.22
- Chapter 6: Figures 6.2, and 6.15 - 6.17

Dr Anthony Beckhouse (formerly of Systems Biology, Eskitis Institute for Cell and Molecular Therapies, Griffith University) assisted with the construction of the tetracycline-inducible AR vector in Chapter 5. Dr Beckhouse contributed advice on cloning methodology, and assistance with cloning procedures in the laboratory.

Dr Grant Stuchbury provided scientific discussion, in addition to proofreading and editing of the thesis. Dr Sabine Fletcher and Dr Amy Jones also assisted in thesis proofreading and editing.
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1 CHAPTER ONE: GENERAL INTRODUCTION
1.1 The Prostate

1.1.1 Anatomy of the Prostate

The prostate gland produces various components of seminal fluid, located near the bladder in males (Mann 1974). Secretory proteins such as prostate-specific antigen (PSA) are synthesised and released into the seminal fluid to support sperm survival (Mann 1974). The prostate is comprised of three morphological regions; the peripheral zone, the transition zone and the central zone (Fig. 1.1 A; McNeal 1969). Whilst age-related conditions of benign overgrowth, including benign prostatic hyperplasia (BPH), tend to occur in the transition zone, prostate cancer primarily arises from the peripheral zone (Greene et al. 1995). The structure of the prostate gland consists of epithelial cells arranged in an acinar structure (Fig. 1.1 B; Abate-Shen et al. 2000). The major components of the prostate epithelium are basal and luminal cells; the basal cells secrete factors that act on the adjacent cells in the luminal layer, whilst the luminal cells actively secrete proteins into the seminal fluid (Abate-Shen et al. 2000).

Figure 1.1 Anatomy of the prostate gland.
A sagittal section of the adult human prostate gland, consisting of the transitional, peripheral and central zones is illustrated in (A). The glandular structure is reflected in the cellular organisation of the prostatic duct (B). A layer of basal cells surrounds the luminal cell population, which secretes proteins into the seminal fluid. Neuroendocrine cells can also be present in the basal cell layer, surrounded by a supportive basal lamina. Figure adapted from Abate-Shen et al (2000).

1.1.2 Development and Maintenance of the Prostate

The development and maintenance of the prostate epithelium are largely mediated by androgens. These male sex steroid hormones are mostly synthesised by leydig cells in the testis and, to a lesser extent, by the adrenal gland (Debes 2002;
The major role of androgens is to regulate the development and maintenance of male sexual characteristics. The survival and growth of prostate epithelial cells is dependent on the presence of androgens; in their absence, the cells undergo apoptosis. The major androgen form, testosterone, is produced in the testis and converted intracellularly to the more potent and active metabolite dihydroxytestosterone (DHT) via the action of the enzyme 5α-reductase. DHT binds to the androgen receptor (AR) in the cell cytoplasm, inducing nuclear translocation and receptor dimerisation (Fig. 1.2). AR then acts as a transcription factor, through a direct interaction with androgen-responsive elements (AREs) in DNA via the DNA-binding domain of the receptor, activating the transcription of target genes (Fig. 1.3; Debes 2002; Heinlein et al. 2004). The binding of androgens to the AR promotes cell differentiation and prostate morphogenesis, as well as regulation of prostate epithelial cell growth and survival (Debes 2002; Heinlein et al. 2004).

Figure 1.2 Activation of the AR by androgens results in the production of PSA.
In its resting state, the AR is bound by heat shock proteins (HSPs) in the cytoplasm. In the presence of androgen ligands such as DHT, HSPs dissociate from the receptor, leaving it free to bind DHT to elicit receptor activation. The AR dimerises and translocates to the nucleus, where it acts as a transcription factor by binding directly to androgen responsive elements (AREs) in DNA. This binding triggers the transcription of AR-target genes, such as PSA, which is then released from the cell through exocytosis, after which it enters the circulation and can be detected in blood. Figure adapted from Heinlein et al (2004).
The ligand-binding domain (LBD) of AR interacts with androgens to initiate receptor activation. Co-activator proteins bind to the amino terminal domain (NTD) of AR, and the DNA-binding domain (DBD) binds to AREs in the promoters of AR target genes. The DBD is joined to the LBD by a hinge region (H). Figure adapted from Centenera et al (2008).

The classical genomic mode of action for AR results in transcriptional activation of genes involved in apoptotic regulation, protein secretion, differentiation and cell growth (Falkenstein 2000; Kaarbo et al. 2007). The widespread effects of androgens are demonstrated by the finding that up to 4.3% of the LNCaP PCa cell line transcriptome can be either directly or indirectly regulated by androgens (Dehm 2006). AR can also elicit effects that do not rely upon changes to transcriptional activity. These non-genomic effects have been associated with the activation of a membrane form of AR (mAR), and include the activation of kinase cascades, calcium flux, focal adhesion kinase (FAK) complexes, cytoskeletal rearrangement and the exocytosis of PSA (Hatzoglou et al. 2005; Kampa et al. 2002; Papakonstanti et al. 2003).

1.1.3 Age-Related Conditions of the Prostate

Abnormal prostate epithelial cell proliferation tends to accompany the ageing process in males. The most common of the age-related prostatic conditions is BPH, whereby an increase in basal and stromal cell proliferation in the transition zone exerts pressure on the urethra and restricts the flow of urine (Abate-Shen et al. 2000). Prostatic intraepithelial neoplasia (PIN) is characterised by the emergence of pre-neoplastic lesions develop due to an overgrowth of abnormal cells. In many cases, PIN can precede the development of PCa (Lee 2008).
1.2 Prostate Cancer (PCa)

PCa is defined as a malignant or cancerous overgrowth of the prostate epithelium, typically occurring in men over the age of 65 (Hsing et al. 2006). It is the most common cancer occurring in Australian men, with one in eleven men developing PCa by the age of 70. In Australia, 20,000 new cases of PCa are diagnosed each year (Prostate cancer statistics: Prostate Cancer Foundation of Australia 2012; Hara et al. 2003). As the second most common cause of cancer deaths in men, close to 3,300 men die of PCa per year; a statistic that exceeds the number of women who die from breast cancer (BCa) annually (Prostate cancer statistics: Prostate Cancer Foundation of Australia 2012).

PCa forms due to a disruption in the normal feedback between androgen stimulation and the balance between cell growth and apoptosis. In the initial stages of PCa, the cancer is confined to the local prostate tissues, and the survival of the tumour cells is dependent on androgens. Most cases of PCa are indolent, slow-growing and are unlikely to cause death in the patient (Abate-Shen et al. 2000). However, some PCas are more aggressive, and rapidly progress towards advanced disease and metastasis. After local invasion of the seminal vesicles, the cancer spreads to establish secondary metastases, primarily in the bone marrow and the lymph nodes (Semenas et al. 2012). Bone metastasis is the major cause of the clinical symptoms and mortality in PCa patients, and mortality rates remain high despite improvements in therapies (Bluemn 2012; Nieto 2007).

The risk of developing PCa is influenced by both genetic and environmental factors, the most significant risk factor being age (Lee 2008). The risk of developing PCa is also enhanced in patients who have experienced prostate inflammation, and those who have a family history of the disease (Crawford 2003; De Marzo et al. 2007; Lee 2008; Nieto 2007).

1.2.1 Stages of PCa Progression

Primary PCa is localised to the prostate gland, causing symptoms including frequent urination, blood in the urine or semen, decreased libido and erectile dysfunction (Symptoms: Prostate Cancer Foundation of Australia 2012). This stage of the disease is effectively treated with radical prostatectomy and/or
external beam radiation. Most primary tumours are cured this way, but some progress to advanced metastatic PCa, leading to mortality (Bluemn 2012).

Advanced PCa is characterised by the spread of the malignant cell populations from the prostate gland. Local invasion of tumour cells into surrounding tissues is followed by metastasis to secondary sites. More noticeable symptoms develop during advanced PCa, including pathologic bone fractures and spinal cord compression as a result of lytic bone metastasis (Picard 2012). The therapy of choice for this stage of disease is androgen deprivation therapy (ADT), through the use of either surgical or chemical castration to reduce the levels of circulating androgens (Bluemn 2012). This is highly effective in halting tumour growth due to the dependence of the tumour cells on androgens for their growth and survival (Chowdhury 2010). PSA levels are monitored during this time as a measure of disease progression. Unfortunately, patients generally develop a resistance to ADT after a median time of 2-3 years, and PSA levels rise again, indicating a progression to castration-resistant PCa (CRPC) (Bluemn 2012; Chowdhury 2010).

CRPC is characterized by a further progression of PCa despite castrate levels of androgens being present in the circulation. Progression to CRPC is often accompanied by metastasis, the symptoms of which include osteoporosis, anaemia and muscle wasting (Perlmutter 2007). Limited treatment options exist for patients at this stage of disease, and the median survival upon progression to CRPC is between 18 and 24 months (Green 2012). The only moderately effective treatment for patients with CRPC is chemotherapy; treatment with docetaxel can improve quality of life, and extend overall survival by up to 3 months (Chowdhury 2010).

1.2.2 Molecular Mechanisms of PCa: Development and Progression

Various molecular changes accompany the development and progression of PCa. Firstly, the loss of chromosomes 10q, 13q or the tumour suppressors phosphatase and tensin homolog (PTEN) and retinoblastoma (Rb) have been noted, and altered expression of cell cycle regulatory genes p27 and p16 are common (Abate-Shen et al. 2000). More recently, studies of DNA copy number and gene expression in 218 prostate tumours have shown the most commonly altered pathways in both primary and metastatic PCa are Rb, PI3K, and Ras/Raf signalling (Taylor 2010).
Progression of PCa to CRPC involves an altered regulation or expression of AR, resulting in a reactivation of AR signalling despite the low level of androgens present in circulation (Taylor et al. 2010).

Possible mechanisms through which AR can be reactivated include enhanced AR expression, AR mutations, AR amplification, AR splice variants, altered recruitment of co-factors, and cross-activation by growth factor pathways (Green 2012; Taylor et al. 2010). Nearly one third of CRPC patients display amplification of the AR locus itself to result in enhanced AR expression (Brown 2002; Edwards 2003; Ford 3rd 2003; Koivisto 1997; Visakorpi 1995). AR mutations have been reported in 8-25% of CRPC tumours, with consequences for AR function (Green 2012). The AR gene was the most commonly mutated gene found in metastatic PCa samples in the comprehensive gene expression study performed by Taylor and colleagues (2010). This study established that alterations in AR through mutation, gene amplification and/or over-expression occurred exclusively in metastatic samples (Taylor 2010). Alterations in specific portions of the AR sequence can confer promiscuous binding, leading to activation of AR by non-androgen steroid hormones (Middleman et al. 1996; Veldscholte et al. 1990). Other mutations can increase the sensitivity of AR to ligand stimulation, or convert the AR antagonists bicalutamide and flutamide to agonists (Hara et al. 2003; Taplin 2004; Veldscholte et al. 1990; Yoshida et al. 2005). Splicing variants of AR have been identified, lacking portions of the carboxy-terminal LBD, conferring constitutive activation of the receptor in the absence of ligand (Dehm et al. 2008; Hornberg et al. 2011; Hu et al. 2009; Jenster 1999; Steinkamp et al. 2009; Sun et al. 2010a). The expression of these variants has been linked with progression to CRPC (Hu et al. 2009; Sun et al. 2010a).

The expression of some AR co-factors is enhanced in recurrent PCa (Agoulnik et al. 2005; Chmelar et al. 2007; Gregory et al. 2001). Taylor and colleagues (2010) reported that many genes in the AR pathway were altered, including several known AR coactivators and corepressors, in 56% of primary, and 100% of metastatic prostate tumour samples.
There is also increasing evidence to indicate the involvement of E-Twenty Six (ETS) fusion genes in PCa progression. The androgen-regulated TMPRSS2 gene can fuse with the ERG oncogene to produce the TMPRSS2-ERG fusion gene, which has been implicated in the progression of PCa (Tomlins et al. 2008). Present in more approximately 50% of prostate tumour samples, TMPRSS-ERG is the most predominant molecular lesion in PCa (Tomlins et al. 2008). Evidence suggests an association between TMPRSS2-ERG and PTEN loss in both murine models of PCa and PCa tumour samples (Carver et al. 2009; King et al. 2009; Taylor et al. 2010; Zong et al. 2009). The expression of this fusion gene can enhance the invasive capacity of PCa tumour cells, and may act to promote the progression of PCa (Tomlins et al. 2008).

Furthermore, AR transcriptional responses can be enhanced in the low-androgen environment of CRPC by cross-activation via various growth factor and kinase pathways (Green 2012). These include epidermal growth factor (EGF), insulin-like growth factor (IGF), interleukin 6 (IL-6) and the Ras-Raf-mitogen activated protein kinase (MAPK), phosphoinosotide 3-kinase (PI3K)/Akt, and human epidermal growth factor receptor 2 (Her2)/neu pathways (Aaronson et al. 2007; Bakin et al. 2003a; Bakin et al. 2003b; Hernes et al. 2004; Krueckl et al. 2004; Mellinghoff et al. 2004; Weber et al. 2004; Yeh et al. 1999). The net effects of these molecular changes to AR activity promote tumour growth, by enhancing the survival and proliferation of PCa cells in low-androgen environments.

1.2.3 Progression to Metastasis

After establishment of the primary PCa tumour, local invasion into the surrounding tissues is followed by the entry of tumour cells into the circulatory system. From here, the tumour cells can migrate to, and establish at, distant sites, leading to the development of a clinically detectable metastatic tumour (Fig. 1.4).
Figure 1.4 The multiple steps towards PCa metastasis.

Tumour cells from the primary prostate tumour make their way through the extracellular matrix (ECM), and enter the circulation via intravasation into blood vessels. From here, the tumour cells adhere to the endothelium at distant sites and exit the circulation via the process of extravasation. The tumour-stromal interactions and the presence of growth factors in the new microenvironment can encourage the growth of the tumour cells, resulting in the formation of clinically detectable metastases. Figure adapted from Sung et al (2007).

The entry of tumour cells into the systemic circulation requires an interaction with endothelial cells lining blood vessel walls during transendothelial cell migration. Adhesive proteins such as integrins and cell surface selectins such as CD11a are known to mediate this process, in combination with the cytoskeletal-regulating Ras-Rho pathway (Clarke et al. 2009). The exit of tumour cells from the circulation is necessary for tumour cells to establish metastasis in tissues at secondary sites in the body.

Cancer metastasis is known to be a non-random process that is heavily influenced by both the expression of adhesion molecules on tumour cells, and the chemoattractants available in specific microenvironments throughout the body (Ben-Baruch 2008). In particular, chemokines and chemokine receptors play a major role in determining the spread, or organ selectivity, of cancer metastasis. Certain niches in the body express high levels of particular panels of chemokines,
whose corresponding receptors may be highly expressed on tumour cells (Ben-Baruch 2008). Chemokines and their receptors work in combination with many other factors to regulate the metastasis of tumours; for example, lipid cells in the bone marrow can also encourage the development of bone metastasis in PCa (Clarke et al. 2009).

Finally, the establishment of secondary cancer metastases at distant sites depends on the factors present in the destination microenvironment. If factors are expressed at the destination site that can encourage tumour cell growth and vascularisation, micrometastases will develop (Bussard 2008; Clarke et al. 2009). In the case of metastasis to the bone, tumour cells invade the bone marrow and disrupt the balance between bone deposition and resorption (Clarke et al. 2009). Subsequent bone destruction can disturb and weaken the structure and function of the bone marrow, resulting in clinically evident symptoms of bone metastasis including bone pain, increased susceptibility to fractures and anaemia (Advanced prostate cancer: Australian Prostate Cancer Collaboration, Inc. 2009; Bone metastasis: American Cancer Society 2012; Clarke et al. 1991; 1993).

1.2.4 Integrins and Cancer Metastasis

Integrin receptors play a major role in regulating both the adhesion of tumour cells to the surrounding ECM, and in mediating interactions with other cell populations in the tumour microenvironment and beyond (Belkin et al. 2000; Bendas et al. 2012). Expressed on the cell surface, these transmembrane glycoproteins occur as heterodimers, comprised of α and β integrin subunits (Belkin et al. 2000; Hynes 2002; Morgan et al. 2007).

In addition to mediating cell-extracellular matrix and cell-cell interactions, integrins act as signalling receptors to transmit information in both directions across the plasma membrane (Bendas et al. 2012; Luo et al. 2007). The affinity of integrins for extracellular ligands is regulated through a process termed "inside-out signalling" (Belkin et al. 2000). Conformational changes in the integrin receptor cytoplasmic domain can be initiated by the intracellular signalling responses of non-integrin receptors, rendering the integrin receptor as capable of recognising a ligand (Belkin et al. 2000; Bendas et al. 2012). In contrast, the "outside-in" signalling occurs through the direct binding of an extracellular ligand to the extracellular domain of the integrin receptor (Belkin et al. 2000).
Conformational changes in the cytoplasmic domain of the integrin receptor are then triggered, permitting the activation of various intracellular signalling pathways including FAK, Src, MAPK and Ras (Aplin et al. 1999; Belkin et al. 2000; Gahmberg et al. 2009; Giancotti et al. 1999; Schoenwaelder et al. 1999; Schwartz et al. 1999). As a consequence, cellular adhesion, proliferation and migration responses are activated, which can mediate adhesion-associated events such as the assembly of basement membranes, tumour cell invasion and metastasis (Belkin et al. 2000).

The specificity of integrin receptors for their ligands can be dictated both by the α/β subunits, and the cellular context of expression (Belkin et al. 2000). To date, 18 α chain subunits and 8 β chain integrin subunits have been characterised (Fig. 1.5; Bendas et al. 2012). As heterodimers, these subunits combine to form 24 unique α/β integrin receptors which are then classified into β1, β2 or αv integrin subfamilies; each with a particular repertoire of ligand binding specificities (Belkin et al. 2000; Bendas et al. 2012).
Figure 1.5 Integrin receptors are integral membrane heterodimers comprised of α and β integrin subunits.

Various combinations of α and β integrin subunits result in the formation of the integrin receptor heterodimer. Connecting lines between α and β subunits indicate binding partners which heterodimerise to mediate cell-cell and cell-ECM adhesion. The combination of these subunits contributes towards the binding specificity of each receptor to ECM proteins such as laminin, fibronectin and vitronectin. A subset of α integrin subunits contain an inserted (I)-domain called von Willebrand Factor, which mediates ligand binding at the α subunit. The α integrin subunits with no I-domain form integrin receptor ligand binding sites that are comprised of both the α and β subunit of the heterodimer. Figure adapted from Gahmberg et al. (2009).

The ability of an integrin receptor to bind its ligand is determined by the conformation adopted by α and β chains in the extracellular integrin receptor domain, and the cell type on which the receptor is expressed (Belkin et al. 2000; Chan et al. 1991; Elices et al. 1989). These factors particularly impact on the ability of the cell to bind ECM proteins including laminin, fibronectin and vitronectin, resulting in variations in cell adhesion properties according to the profile of integrin receptors expressed on the cell surface (Witkowski 1993). Due to their central role in mediating cell adhesion to the ECM, integrin receptors strongly contribute to the regulation of tumour cell invasion and metastasis (Belkin et al. 2000; Castronovo 1993; Witkowski 1993).
1.3 Clinical Aspects of PCa: Diagnosis and Treatment

1.3.1 Diagnosis

Men over 50 years of age are recommended to undergo regular screening for PCa via a combination of digital rectal examination (DRE) and the PSA test (Crawford 2003). The DRE can be used to manually assess the presence of a clinically evident prostate tumour (Cheng 2012). The PSA test is based on the analysis of PSA concentration in the blood, the production of which is known to increase with prostate volume, and can indicate the presence of PCa (Crawford 2003). Patient levels are measured against established guidelines, and further investigations are prompted if designated age group-specific thresholds are exceeded. In general, a PSA reading of up to 10 ng/ml can indicate either BPH or localised cancer and more than 30 ng/ml indicates advanced PCa (Testing and diagnosis: Prostate Cancer Foundation of Australia 2012). An abnormal result from these tests can then lead to the formal diagnosis of PCa through a prostate biopsy, after which the sampled tumour can be staged and graded according to the level of malignancy and tissue invasion. The expression of PSA is not specific to malignancy, and therefore the use of the PSA test as a tool for PCa diagnosis is limited. Readings can be elevated due to either prostate inflammation and cancer (Barqawi 2012; Shariat 2011). The limitations of the PSA test are discussed in more detail in section 1.3.3.

PCa tissue is known to display a high level of heterogeneity, thereby complicating the process of diagnosis. In the one sampled lesion, various stages of cancer can be present, sometimes in combination with benign inflammatory conditions. The standard approach utilised by pathologists for grading PCa is the Gleason method, which takes this heterogeneity into account by giving an overall score based on the sum of the two most prevalent grades of neoplastic foci (Gleason 1992). The Gleason method is recognised as a valuable tool for PCa prognosis, in which a higher Gleason score indicates more advanced PCa (Fig. 1.6; Table 1.1; Abate-Shen et al. 2000; Gleason 1992).
A Gleason grade of 1-5 is assigned according to the level of histological change in malignant prostate biopsies. Patient biopsy samples containing cancer cells residing in tissue with similar appearance to normal glandular tissue are assigned a low Gleason grade (1-2). A moderate Gleason grade (grade 3) is assigned when more abnormalities are present, with multiple small glandular spaces still remaining. For patient biopsies which display highly abnormal tissue architecture that no longer resembles normal prostate tissue, a high Gleason grade (4-5) is assigned. Figure adapted from Banas et al. (2010).

<table>
<thead>
<tr>
<th>Gleason Score</th>
<th>Aggressiveness of PCa</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4</td>
<td>Low</td>
<td>Relatively slow growing and not likely to be aggressive</td>
</tr>
<tr>
<td>5-6</td>
<td>Moderate</td>
<td>Fast growing, moderately aggressive</td>
</tr>
<tr>
<td>7</td>
<td>Intermediate</td>
<td>Fast growing, moderately aggressive</td>
</tr>
<tr>
<td>8-10</td>
<td>High</td>
<td>Aggressive cancer</td>
</tr>
</tbody>
</table>

Table adapted from Staging and grading: Prostate Cancer Foundation of Australia (2012).

The extent to which the cancer has progressed or metastasised can be further analysed using the Tumour, Node and Metastasis (TNM) staging system (Table 1.2; Cheng 2012). Following positive diagnosis of PCa via biopsy, imaging techniques such as bone scans, computed tomography (CT) or magnetic resonance imaging (MRI) may be employed to determine the extent of disease and any skeletal spread (Staging and grading: Prostate Cancer Foundation of Australia 2012). Based on this combination of diagnostic tools, a patient treatment regime is then formulated.
Table 1.2 Staging of PCa biopsy tissue samples using the Tumour Node and Metastasis (TNM) staging system.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Progression of Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>The cancer is small and contained within the prostate</td>
</tr>
<tr>
<td>T2</td>
<td>The cancer is larger and may be in both lobes of the prostate, but is still confined to the organ</td>
</tr>
<tr>
<td>T3</td>
<td>The cancer has spread beyond the prostate and may have invaded adjacent lymph glands or seminal vesicles</td>
</tr>
<tr>
<td>T4</td>
<td>The cancer has spread to other organs, or to bone</td>
</tr>
</tbody>
</table>

Table adapted from Cheng et al. (2012).

1.3.2 Current PCa Treatment Options

The course of action for patient treatment is dependent on the stage of disease progression at the time of diagnosis, patient preference and quality of life issues (Picard 2012). Treatment options range from active surveillance to surgical prostate removal (radical prostatectomy), chemical castration, hormone therapy, chemotherapy and radiotherapy (Picard 2012).

Cancer localised to the prostate in the early stages of disease is usually treated by radical prostatectomy and/or radiation (Abate-Shen et al. 2000). Many cases of PCa are relatively indolent and slow-growing, and do not progress to cause mortality in patients. Indeed, when the cancer is deemed low risk and is localised to the prostate, active surveillance is recommended; where a regular PSA screening and prostate biopsy is undertaken to monitor whether disease has progressed. Upon disease progression, more aggressive treatment options can then be employed (Picard 2012). However, it is difficult to distinguish these indolent tumours from more aggressive cases in the clinic, and the course of action is usually based on patient preference (Post diagnosis: Prostate Cancer Foundation of Australia 2012).

The great hurdle to PCa treatment and management is the progression to CRPC and advanced metastatic disease. The options for treatment at this stage of disease are aimed at improving quality of life and overall survival. Generally, ADT is combined with other treatments including chemotherapy or radiation (Perlmutter 2007). ADT can be achieved through either surgical or medical castration; medical castration involves administration of a luteinising hormone releasing
hormone (LHRH) agonist or gonadotropin-releasing hormone (GnRH). In particular, LHRH binds to the LHRH receptor in the pituitary, triggering a transient rise in serum luteinising hormone (LH) and testosterone. After this initial increase, the secretion of both LH and testosterone are inhibited, and serum levels of testosterone are reduced (Picard 2012). Early administration of ADT provides quality of life benefits, including a reduction in pathologic fractures, spinal cord compression and urinary obstruction. However, it did not benefit overall survival (Picard 2012). ADT is initially effective in the treatment of advanced PCa, resulting in improvements to cancer-related symptoms and tumour regression (Green 2012). Further enhancement of androgen blockade can be achieved by combining LHRH agonists with anti-androgens such as bicalutamide, which prevent actions of adrenal androgens on the AR (Perlmutter 2007; Picard 2012). Combining these treatments has been demonstrated to increase overall survival by 7 months, when initiated after diagnosis of metastatic PCa (Perlmutter 2007). More recently, a competitive AR antagonist called MDV3100 has been shown to display improved affinity and efficacy against AR activation and transcriptional activity compared to Bicalutamide (Tran et al. 2009). Administration of MDV3100 to patients with CRPC in phase I/II trials have shown favourable outcomes for patient disease progression measurements (Scher et al. 2010).

Standard cancer treatments, such as chemotherapy and radiotherapy, are also used for the treatment of PCa. Radiotherapy is usually employed for the treatment of localised high risk PCa, and phase III clinical trials have shown an increased benefit from combination with the LHRH agonist, goserelin (Picard 2012). These therapies together provided a 50% reduction in risk of death due to PCa, improvements to disease-free survival, biochemical free survival and in some cases improvements to distant metastasis over 5-10 year follow-up period (Picard 2012). Patients showing progression to CRPC can rapidly progress towards mortality, and treatment with docetaxel-based chemotherapy can extend median overall survival by approximately 2 months, also improving quality of life (Picard 2012). Further treatment options for patients with advanced metastatic disease are under investigation to improve disease-free and overall survival, particularly focussing on combinatorial treatment approaches (Picard 2012).
1.3.3 Over-Diagnosis and Over-Treatment of PCa

The PSA test is the leading tool used for early PCa detection, however the use of this test as a diagnostic tool is complicated by the fact that it is not specific for malignancies (Barqawi 2012; Shariat 2011). PSA is produced by all prostate epithelial cells and indeed can reflect the presence of prostate malignancy due to an increased volume of the gland. However, abnormal PSA readings can also be caused by other prostate conditions such as BPH, infection or chronic inflammation (Shariat 2011). In addition, the PSA levels can naturally vary between individuals, meaning the designated thresholds for diagnosis are not always applicable. The limited relevance of PSA as a screening tool in PCa was emphasised by one particular study showing that age-specific PSA cut-offs missed 20-60% of cancers in men over 60 years of age (Catalona 2000). Collectively, these factors have contributed towards an over-diagnosis of PCa; the diagnosis of a cancer that would not otherwise go on to cause symptoms or mortality (Klotz 2012). Early detection of PCa enables early intervention, however due to the non-specific nature of the test it is difficult to distinguish patients at high risk of disease progression from those with a low risk of progression (Barqawi 2012). Clinically insignificant cancer is defined by slow growing cancer that does not produce noticeable symptoms, and ideally, active surveillance would be prescribed for these cases. Using the D’Amico criteria, clinically insignificant PCa are low PSA (<10 ng/ml), a Gleason score of 6 or less, and a T1 clinical stage (Barqawi 2012). However, no general consensus has been reached on defining clinically insignificant cancer, leading to confusion and prescription of more aggressive treatment options (Barqawi 2012; Shariat 2011). Consequently, patients endure unnecessary side effects from these treatments that impact on quality of life (Table 1.3). One way in which this problem is being addressed is through the study and development of more reliable and specific biomarkers for PCa.
1.3.4 Novel Biomarkers for PCa

The lack of specificity and accuracy of the PSA test as a screening tool has prompted research into the development of more effective PCa biomarkers that can be measured in the blood or urine. The field of PCa-specific biomarkers is expanding rapidly, with an increasing interest directed at the identification of prognostic biomarkers that may provide information on disease outcome (Armstrong 2012). Such biomarkers may help to distinguish indolent from aggressive PCa, and to identify progression to CRPC. Furthermore, clinical studies are being undertaken to determine whether selected biomarkers may show value in directing treatment options for patients with a positive PCa diagnosis (Armstrong 2012). Other markers under investigation include predictive biomarkers, to indicate the likelihood of response to a particular therapeutic approach, and surrogate markers to serve as early indicators of survival outcomes in the clinic prior to patient mortality (Armstrong 2012). Many candidate biomarkers show prognostic ability, but there are few candidates for predictive or surrogate markers.

Rather than the currently employed PSA test which measures total PSA, levels of specific PSA subtypes and forms may be more effective at indicating aggressive pathologic features (Shariat 2011). PSA derived forms include total, intact and free PSA (fPSA); with percentage of fPSA showing enhanced specificity and sensitivity to cancer detection (Catalona 2000). Of the three types of fPSA identified, the percentage of pro-PSA can improve specificity for cancer detection,
and can more selectively detect aggressive PCas of Gleason 7 grade and above (Chmelar et al. 2007; Gregory et al. 2001). Additional biomarkers with potential to identify and diagnose PCa include early PCa antigen (EPCA), transforming growth factor β (TGFβ), IL-6 and E-twenty six (ETS) fusion genes. Some of these biomarkers are being employed in clinical trials, and are under various stages of development (Table 1.4) (Shariat 2011).

Other biomarkers are being developed for the early detection of bone metastasis in advanced PCa (Dehm et al. 2008). Current diagnostic approaches to the detection of bone metastasis rely on imaging techniques, which display limited sensitivity and can restrict the potential detection of bone metastasis at early stages. Rather, biomarkers that may enable earlier detection of bone metastasis are under investigation (Shariat 2011). Clinical trials have shown that levels of various bone metabolism and turnover markers are elevated in patients with bone metastasis (Table 1.4). All prognostic biomarkers in clinical use show value when measured prior to starting therapy, and only circulating tumour cells (CTCs) are being studied as prognosis, predictive and surrogacy biomarkers (Shariat 2011). Rather than use of biomarkers in isolation, it has been suggested that a panel of biomarkers may be of better use to guide clinical decisions (Shariat 2011).
### Table 1.4 PCa Biomarkers under investigation for clinical development.

<table>
<thead>
<tr>
<th><strong>Indication</strong></th>
<th><strong>Biomarker</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Predict PCa Occurrence</td>
<td>EPCA</td>
<td>(Dhir <em>et al.</em> 2004)</td>
</tr>
<tr>
<td>Distinguish between men with and without PCa</td>
<td>Percentage of free PSA</td>
<td>(Catalona 2000)</td>
</tr>
<tr>
<td></td>
<td>hK2</td>
<td>(Becker <em>et al.</em> 2000; Haese <em>et al.</em> 2001)</td>
</tr>
<tr>
<td></td>
<td>uPA</td>
<td>(Gupta <em>et al.</em> 2009; Hienert <em>et al.</em> 1988; Miyake <em>et al.</em> 1999; Shariat <em>et al.</em> 2007)</td>
</tr>
<tr>
<td></td>
<td>PCA3</td>
<td>(Deras <em>et al.</em> 2008; Hessels <em>et al.</em> 2007; Marks <em>et al.</em> 2007)</td>
</tr>
<tr>
<td></td>
<td>CTCs</td>
<td>(Shariat 2011)</td>
</tr>
<tr>
<td>Indicate stage of PCa progression, predict aggressive PCa or metastasis</td>
<td>EPCA</td>
<td>(Dhir <em>et al.</em> 2004)</td>
</tr>
<tr>
<td></td>
<td>TGFβ</td>
<td>(Ivanovic <em>et al.</em> 1995; Shariat <em>et al.</em> 2004; Shariat <em>et al.</em> 2001)</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>(Michalaki <em>et al.</em> 2004; Nakashima <em>et al.</em> 2000)</td>
</tr>
<tr>
<td></td>
<td>ETS fusion genes</td>
<td>(Attard <em>et al.</em> 2008a; Laxman <em>et al.</em> 2006; Mosquera <em>et al.</em> 2009; Tomlins <em>et al.</em> 2005)</td>
</tr>
<tr>
<td></td>
<td>Percentage of pro-PSA</td>
<td>(Catalona <em>et al.</em> 2003; Catalona <em>et al.</em> 2004)</td>
</tr>
<tr>
<td></td>
<td>CTCs</td>
<td>(Shariat 2011)</td>
</tr>
<tr>
<td>Bone metastasis</td>
<td>Alkaline phosphatase</td>
<td>(Coleman <em>et al.</em> 2005; Schindler <em>et al.</em> 2008)</td>
</tr>
<tr>
<td></td>
<td>PINP and PICP</td>
<td>(Zissimopoulos <em>et al.</em> 2008; Zissimopoulos <em>et al.</em> 2009)</td>
</tr>
<tr>
<td></td>
<td>Osteopontin</td>
<td>(Carlinfante <em>et al.</em> 2003; Ramankulov <em>et al.</em> 2007)</td>
</tr>
</tbody>
</table>

**Abbreviations:** hK2 – human kallikrein 2; EPCA – Early PCa antigen; uPA – Urokinase plasminogen activation axis; AMACR – α-Methylacyl-CoA racemase; PCA3 – PCa antigen 3; CTC – Circulating tumour cell; PINP and PICP - N-terminal propeptide of pro-collagen type 1 and C-terminal propeptide of pro-collagen type 1, respectively. Table adapted from Armstrong *et al.* (2012), Shariat *et al.* (2011), Wang *et al.* (2005b).
1.4 Models of PCa: *In Vivo* and *In Vitro* Approaches

The effectiveness of treatment and the establishment of potential cancer biomarkers can be elucidated at a molecular level using basic research. The current models for cancer research are based around *in vivo* whole animal models, or *in vitro* approaches including the traditional 2D cell culture monolayer versus the more recently developed 3D culture model (Fig. 1.7; Nyga et al. 2011).

![2D Culture and 3D Culture](image)

Figure 1.7 *In vitro* models used for cancer research.

Traditional *in vitro* culture of cancer cell lines is based on growing cell monolayers on a tissue culture-treated plastic or glass surface. The use of 3D matrices for culturing of cancer cells facilitates the formation of spheroid structures within a supportive extracellular matrix (ECM). Figure adapted from Zschenker et al. (2012).

The structure of epithelial tissue is comprised of polarised apical and basal surfaces, positioned on a basement membrane of ECM proteins, such as collagen IV or laminin. The epithelium within secretory structures, such as the prostate or breast, display a glandular organisation. Epithelial cells form a spherical acinar structure surrounding a hollow lumen (Yamada 2007). Highly proliferative basal epithelial cells comprise the outer layer, and secretory luminal epithelial cells line the internal region of the structure (Fig. 1.1 B; Abate-Shen et al. 2000; Feder-Mengus et al. 2008).

The tumour microenvironment is comprised of tumour cells and supporting stromal cells, surrounded by basement membrane and ECM (Bussard 2008; Sung et al. 2007). Various interactions occur between these components to support the growth and development of the tumour to a clinically detectable size (Bussard...
2008; Sung et al. 2007). This is more closely recapitulated in 3D cell culture than traditional 2D monolayer culture.

1.4.1 Traditional 2D Cell Culture

2D culture is the growth of cells as a monolayer on a plastic or glass vessel. This method is used to study the behaviour of cells in the normal physiological, developmental and malignant states. Two-dimensional culture has provided insights into molecular mechanisms regulating the growth and behaviour of tumour cells. Using this approach, conditions are more easily manipulated and can be effectively used to investigate pathway modulation in isolation of other factors. However, the convenience of 2D culture is balanced by the limitations of this approach in representing the characteristics of tumours cells observed in vivo. A loss of cell polarity, cell-cell contacts and cell-ECM interactions is observed in 2D cultures (Harma et al. 2010). Evidence of a strong genetic adaption to 2D culturing conditions was presented in a study by Birgersdotter and colleagues (2005). Gene expression patterns in cell lines grown in 2D culture were compared to those in their corresponding tumour tissues in vivo. Patterns of gene expression were estimated to differ by up to 30%; specifically, genes regulating cell cycle regulation and DNA repair and metabolism. Genes that were involved in splicing, translation and protein modification were also affected (Birgersdotter 2005).

To more effectively maintain tumour cell characteristics and behaviours, whole animal models have been utilised as an in vivo approach for cancer research. Generally, mouse xenograft models are utilised for this purpose, in addition to genetically engineered mouse (GEM) models (Nyga et al. 2011; Richmond 2008). Xenograft models utilise immunocompromised mice, which are implanted with primary cells or cell lines, either subcutaneously or directly into a particular organ (Nyga et al. 2011). After injection into the mouse model, tumour growth and development can be observed over a 1-8 week period (Nyga et al. 2011). Whole-animal imaging can be used to monitor tumour growth, or more commonly, tumour volume can be inspected in the sacrificed animal (Nyga et al. 2011). Alternatively, GEM models can be modified to display altered expression of malignancy-associated genes, through mutation, deletion or over-expression (Richmond 2008). Knockout mice are a commonly used GEM model used in cancer research (Richmond 2008).
1.4.2 3D Cell Culture in Cancer Research

As an alternative to 2D cell culture and the whole-animal in vivo studies, a more physiologically relevant in vitro model has been established using 3D cell culture methods. Three-dimensional culture is broadly defined as an aggregation of cells into a 3D spatially oriented structure, using a variety of different approaches. This method has been used in studies of tissue engineering, embryonic development, physiology and has also been employed in the field of cancer research (Zschenker et al. 2012).

The culturing of tumour cells in 3D encourages the formation of a spheroid structure, consisting of actively proliferating cells bordering an inner, nutrient deprived zone towards the centre of the mass (Fig. 1.8; Nyga et al. 2011). The aggregation of cells can be encouraged either in suspension or by culturing cells on a layer of ECM or protein scaffold (Feder-Mengus et al. 2008). Spheroid formation requires cell-cell interactions and cell polarity, and also contact with the surrounding ECM. Spheroid formation also results in the emergence of a gradient of nutrient and oxygen deprivation towards the centre of the mass. This gradient is seen to be more reflective of tumour biology, in which a starved population of cells is present in the centre of the structure, and is more physiologically relevant than a fully nourished monolayer of cells in 2D culture (Nyga et al. 2011; Yamada 2007). Furthermore, spheroid formation is accompanied by a deposition of basement membrane or basal lamina around the mass, another characteristic that is observed in vivo and not in 2D culture. Through these characteristics, 3D spheroids can more effectively recapitulate the structure of tumours in vivo when compared to traditional 2D monolayer culture approaches.
3D culture methods that utilise ECM constituents facilitate the interaction of cells with their surroundings via cell-ECM contacts, which can have profound effects on cell growth and phenotype (Fischbach et al. 2009; Nyga et al. 2011; Zschenker et al. 2012). The method of growing cancer cells in suspension through slow rotation at 37°C permits an aggregation of tumour cells, but lacks cell-ECM interaction. It is now known that interaction of cells with the basement membrane and ECM can regulate cell behaviour through modulating cell growth and survival, cell migration, attachment and dissemination (Nyga et al. 2011; Zschenker et al. 2012). Commercially available ECM preparations for use in 3D culture can be derived from the basement membrane deposited by cells in vitro; Matrigel (BD Biosciences), in particular, is a laminin-rich ECM preparation derived from engelbreth-holm-swarm (EHS) tumour cells (Hughes 2010). Other methods of 3D culture involve the use of natural or synthetic scaffolds to support the growth of spheroids in vitro, and contain a more precisely defined composition to cell-derived ECM. The scaffold is comprised of natural or synthetic proteins that form intertwined fibres onto which cells are seeded. Spheroids are formed in the spaces between the fibres. The composition of natural scaffolds tends to be ECM proteins such as collagen or laminin, which permit the study of cell migration or invasion. Synthetic scaffolds include those made from
polylactide (PLA), polyglycolide (PGA) and co-polymers such as poly(lactide-co-glycolide) (PLG/PLGA), which are biodegradable and can generate a mesh, fibre or sponge (Nyga et al. 2011). These synthetic scaffolds require coating with adhesion peptides to facilitate cell adhesion (Kim 2005; Nyga et al. 2011). Scaffold constituents and adhesion proteins can be selected to provide a specific environment, and can be readily manipulated, to exert a more precise control over the microenvironment (Kim 2005; Nyga et al. 2011).

The use of Matrigel to generate 3D cultures of tumour cells permits the formation of spheroids with distinct morphological characteristics in vitro. In a study by Kenny and colleagues (2007), a panel of normal breast epithelial and BCa cell lines were cultured in Matrigel and their morphological features were assessed after 4 days in culture into round, mass, grape-like or stellate (Fig. 1.9).

![Figure 1.9 The development of distinct spheroid morphologies in 3D culture.](image)

A large panel of 25 breast (normal epithelial and cancer) cell lines were reported to form four main morphologies after 4 days of growth in Matrigel: Round, mass, grape-like or stellate. The round morphology consisted of organised nuclei and robust cell adhesion, whereas the mass phenotype displayed disorganised nuclei. The grape-like phenotype showed poor cell adhesion, whereas the stellate phenotype displayed invasive processes protruding from the spheroid mass into the surrounding Matrigel matrix. Figure adapted from Kenny et al (2007).

The round phenotype was established by non-invasive cell lines, whereas the grape-like and mass phenotypes were formed by cell lines with higher invasive and metastatic potential (Kenny 2007). The stellate morphology, in which filopodia were seen to protrude out into the ECM, was associated with the most invasive phenotype in vitro (Kenny 2007). A similar study was performed by Harma and colleagues (2010) for 29 prostate cell lines, including prostate epithelial and PCa cells. Some cell lines formed round, well-defined spheroid structures (prostate epithelial cells PrEc, RWPE-1, DU145) however the majority of PCa cell lines formed large irregular mass spheroids (LNCAP, LNCAP C42b, MDA PCa1), as did some of the transformed normal prostate epithelial cells (22rv1; Harma et al. 2010). Some cell lines formed a stellate phenotype in which
spindle-like filopodia formed invasive structures that rapidly migrated through the surrounding ECM (PC3, PC3M, ALVA 31; (Harma et al. 2010). Other cell lines formed loose, grape-like spheroids (LAPC-4), and other cultures consisted of single cells which did not form spheroids (VCaP, MDA PCa 2b; (Harma et al. 2010).

Through microarray analysis of mRNA expression in these cell lines, it was shown that culturing in 3D was generally associated with changes to genes that regulate metabolism, epigenetic reprogramming, and ECM remodelling (Harma et al. 2010). Reduced proliferation in 3D was evidenced by down-regulation of mitochondrial/ribosomal function and genes regulating various pathways of cellular metabolism. In addition, the morphological groupings of these cell lines corresponded with altered expression of gene clusters (Harma et al. 2010). Whilst the round spheroid phenotype was associated with basal-like phenotype rich in basal keratin expression (keratin 5, keratin 13), the mass phenotype expressed genes related to luminal differentiation and androgen-inducible genes (cytokeratin 18, NK3 homeobox 1, kallikrein-related peptidase 4; (Harma et al. 2010).

The emergence of the more invasive stellate phenotype in other cell lines was strongly associated with the induction of genes related to cell adhesion, cell-cell contact, invasion/metastasis and ECM turnover (Harma et al. 2010). In particular, an up-regulated expression of integrins (α10, β4, β2), laminins and collagens were observed. Also, the cell lines which displayed a stellate phenotype expressed higher basal levels of mesenchymal markers (vimentin, fibronectin-1, neural(N)-cadherin, cadherin-11) and loss of both the epithelial marker E-cadherin, and epithelial-to-mesenchymal transition (EMT)-related transcription factors (Harma et al. 2010).

1.4.3 3D Culture as a Tool for Studying Cancer Biology

Useful insights into tumour biology have been gained into the resistance of tumour cell masses to chemotherapy and radiotherapy using 3D culture methods. Spheroids generated in 3D culture display an enhanced resistance to radiotherapy and chemotherapy, a property consistent with tumour masses in vivo (Zschenker et al. 2012). Tumour cell spheroids also display decreased sensitivity to drug-induced apoptosis, and the cytostatic or cytotoxic effects of particular
chemotherapeutic agents (Feder-Mengus et al. 2008; Zschenker et al. 2012). When the mechanism of this resistance was investigated in 3D cultures, modulation of genes clustered in functions of regulating ECM, cell adhesion and defence response were associated with the resistant phenotype (Zschenker et al. 2012). In particular, differential expression of genes that regulate integrin signalling, cell shape and cell-cell contact were found to contribute towards this resistance (Zschenker et al. 2012).

The function of integrin β1 has been implicated in the resistance of BCa cells to other targeted therapies (Dehm et al. 2008). The growth of tumours in some BCa patients can be effectively controlled with Her2-targetted therapies such as Ilpatinib and/or trastuzumab, however other patients develop resistance to these therapies (Dehm et al. 2008). In the absence of Her2 activity, activation of integrin β1 has been shown as an alternative pathway to regulate cell growth, possible serving as a mechanism of resistance to these therapies (Dehm et al. 2008). Indeed, 3D culturing has highlighted a role for cell-ECM interactions and, in particular, integrin function in regulating growth and survival of cancer cells. Inhibition of integrin β1 function negatively impacted on the proliferation and apoptosis of BCa cell lines (Weaver et al. 1997), a result that was mirrored in studies of PCa cells cultured in 3D (Goel 2010). Furthermore, the morphology of PCa spheroids was affected by integrin β1 inhibition, causing a reversion towards the normal epithelial phenotype, and enhanced cell-cell contacts in PC3 cultures (Goel 2010). Studies utilising synthetic 3D scaffolds have shown that engagement of integrins expressed on tumour cells can regulate angiogenic signalling (Fischbach et al. 2009). Taken together, these studies show that cell-ECM interactions via integrins can impact on various facets of cancer cell growth and behaviour.

To date, 3D cultures have been used to model cancer cell aggregation, migration, proliferation, release of angiogenic factors and formation of hypoxic central region of the tumour mass (Nyga et al. 2011). To enhance the ability of these models to reflect tumour tissues in vivo, further research is required into the optimal matrix components, and incorporation of mechanical stress or fluid flow into these models (Fig. 1.10; Yamada 2007). Although the precise tumour environment found in vivo is not completely understood and therefore cannot yet
be completely recapitulated \textit{in vitro}, 3D culture is a more physiologically relevant model that is showing promise in bridging the gap between \textit{in vitro} and \textit{in vivo} approaches (Yamada 2007).

![Diagram of factors in the tissue microenvironment](image)

**Figure 1.10 Factors in the tissue microenvironment can influence cell behaviour.** Various spatial and temporal aspects can impact on cell behaviour \textit{in vivo} including the availability of growth factors, the interaction of cells with the surrounding ECM, supply of nutrients, and fluid flow. Arrows indicate the effects that cells can exert on their environment. Figure adapted from Yamada et al (2007).

### 1.5 Therapeutic Targets in PCa

#### 1.5.1 Androgens and the AR

Although AR was previously considered to only play a role in regulating growth of PCa at the early stages of disease, it is now known to influence the growth of tumour cells in CRPC. Clinically, high levels of AR expression and PSA production are observed in patients with CRPC (Bubendorf et al. 1999; Ford 3rd 2003; Mohler et al. 2004; Ryan et al. 2010). Patients at this stage of disease have been shown to benefit from ADT, and steroid biosynthesis inhibitors are highly effective in CRPC (de Bono et al. 2011; Green 2012; Reid et al. 2010). The findings of Taylor and colleagues (2010) that altered expression of genes in the AR pathway are found in 100% of metastatic PCa samples also supports a role for AR pathway involvement in late-stage PCa. Collectively, those observations strongly indicate androgens and the AR play an active role in advanced PCa and CRPC, showing that this axis is therefore a solid target for intervention in all stages of PCa (Green 2012).
One approach to AR-targeted therapies is the improvement of AR antagonists to display higher receptor affinity, without the agonist ability displayed by bicalutamide and flutamide (Green 2012). One particular compound, MDV3100, is a competitive AR antagonist that binds the AR with higher affinity than bicalutamide (5-8-fold), inhibits AR nuclear translocation and chromatin occupancy at canonical AREs (Tran et al. 2009). Administration of MDV3100 in CRPC patients prior to docetaxel during phase I/II studies have shown more than a 50% decline in PSA, and significant changes to the median time to PSA progression (Scher et al. 2010). Another AR antagonist, EPI-001, is currently under development for phase I/II studies (Green 2012). This antagonist functions by binding to the amino terminus of the AR receptor, which does not affect ligand binding, but is necessary for activation of AR and recruitment of AR co-activators (Andersen et al. 2010).

Another approach to targeting the androgen axis is the use of steroid biosynthesis inhibitors. The intra-tumoral synthesis of androgens by malignant cells can assist in the survival of PCa cells under castrate conditions, through their expression of steroidogenesis-promoting enzymes (Montgomery et al. 2008; Stanbrough et al. 2006). Therefore, inhibitors that target this steroidogenesis pathway are being developed in the clinic (Green 2012). A promising candidate has arisen in the form of Abiraterone acetate, a drug which selectively, potently and irreversibly inhibits the key steroid biosynthesis enzyme cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) in the androgen biosynthesis pathway (Attard et al. 2009; de Bono et al. 2011). In men with CRPC, administration of Abiraterone acetate can reduce PSA and circulating levels of androgens (Attard et al. 2009; Attard et al. 2008b; Ryan et al. 2010). It has shown success in phase I/II trials, either alone or following docetaxel-based chemotherapy (Attard et al. 2009; de Bono et al. 2011). So far, in phase III clinical trials of men with metastatic CRPC, Abiraterone administered after docetaxel has resulted in a 36% improvement in median overall survival (14.8 vs. 10.9 months) and 35% reduction in risk of death (de Bono et al. 2011). Additional steroid biosynthesis inhibitors being pursued in phase I/II clinical trials include TAK700 and VN/124-1 (TOK-001) (Green 2012).
1.5.2 Chemokines and Chemokine Receptors

1.5.2.1 The Functions of Chemokine Receptors

Chemokines are chemotactic cytokines which are small proteins (8-12kDa) produced by cells and released into the extracellular space to co-ordinate the response of various cell types in the bloodstream or tissues (Blanpain et al. 2003; Wells et al. 1998). A gradient of chemokines can facilitate the directional chemotaxis of cells which express the appropriate chemokine receptors (Oppermann et al. 1999). All chemokines induce the migration of immune cells, and some serve as chemoattractants as part of the inflammatory response by regulating transendothelial migration (Ebnet et al. 1999). Other chemokines are constitutively expressed at lymphoid organs, and regulate the homeostatic processes of embryonic development, angiogenesis and haematopoiesis (Ben-Baruch 2008; Murphy et al. 2000; Neel et al. 2005; Wells et al. 1998).

Expression of chemokines and their receptors are tightly regulated and cell type-specific, however their functions display a large degree of redundancy (Fig. 1.11; Busillo et al. 2007; Wang et al. 1998; Wells et al. 1998). The chemokines are classified according to their amino acid sequence, whereby the position of conserved amino-terminal cysteine residues dictates their assignment to a particular class; C, CC, CXC or CX₃C (where C indicates the conserved cysteine and X indicates any other amino acid) (Murphy et al. 2000). This structural classification can relate to chemokine functionality; CXC chemokines generally act on polymorphonuclear cells such as neutrophils, whereas CC chemokines typically attract monocytes, basophils, eosinophils, T cells, Natural killer (NK) cells and dendritic cells (DC) (Brigati et al. 2002). Chemokines and their receptors are known to be promiscuous and redundant. Many of these receptors bind more than one chemokine, and the majority of chemokines can activate more than one receptor (Fig.1.11; Ben-Baruch 2008; Vindrieux et al. 2009). Not only does this allow for compensation of functionality, but has been linked to the ability to co-ordinate responses according to type of stimuli, intensity, localisation and timing (Ben-Baruch 2008).
The redundancy and promiscuity of the chemokine network.

The chemokine network is largely comprised of many overlapping receptor-ligand specificity. In most cases, more than one chemokine can bind to one receptor (“shared” receptors). There is a small group of specific receptors that have to date been reported to bind one ligand only (“specific” receptors). A subgroup of chemokine receptors that bind to their ligands without signalling activation are in a third grouping (“Decoy” receptors). Chemokine ligands relevant to this thesis are CXCL12 (Stromal-derived factor 1-alpha or SDF-1α), CXCL11 (Interferon-inducible T cell alpha chemoattractant or ITAC) and CXCL8 (interleukin-8 or IL-8). Figure adapted from Sun et al (2010b).

Chemokine receptors belong to the large family of rhodopsin-like, seven-transmembrane G protein-coupled receptors (GPCRs). This association with G proteins links activation of chemokine receptors with an intracellular signalling response (Fig. 1.12; Vindrieux et al. 2009). The scale and involvement of different pathways can differ between tissues, cell types, physiologic or pathologic conditions (Vindrieux et al. 2009). Typically, receptor stimulation causes activation of the PI3K pathway, phospholipase C (PLC) and calcium mobilisation (Krueckl et al. 2004). Other pathways that can be activated include activation of extracellular signal-regulated kinase 1/2 (ERK 1/2), p38 MAPK, Ras, the Rho family of GTPases, p21 activated kinase and nuclear factor kappa B (NF-κB) to trigger cell differentiation, cell migration, survival and proliferation (Thelen 2001). Some “decoy” chemokine receptors bind their ligand with high affinity but do not activate signalling pathways. These receptors may sequester excess chemokines under certain conditions to control chemotactic gradients in tissues (Vindrieux et al. 2009).
Chapter One: General Introduction

Introduction

Figure 1.12 Signalling pathways downstream of chemokine receptor activation.
The binding of chemokines to their receptors can elicit activation of numerous intracellular
signalling pathways. The formation of the FAK complex can stimulate Rho GTPases to drive cell
adhesion and migration. Janus activated kinase-signal transducer and activator of transcription
(JAK-STAT) and can activate the PI3K/Akt signalling pathway. The association of β-arrestin with
the chemokine receptor can lead to activation of the MAPK cascade. Activation of the
Ras/Raf/MEK/ERK pathway as part of the MAPK signalling cascade can result in gene
transcription. Cellular functional responses to stimulation include cell migration, survival and
proliferation. Figure adapted from O’Hayre et al (2008).

1.5.2.2 Chemokines and Cancer

Many different tumour cell types display up-regulated expression of chemokines
and chemokine receptors. As this network can mediate chemoattraction, cell
migration, growth, survival and angiogenesis, this expression profile can confer
an advantage to tumour cells. Expression of particular chemokines and their
receptors has been associated with many facets of tumour development and
progression (Ben-Baruch 2008; Singh et al. 2011). Through their actions on
tumour cells, immune cells, stromal cells and the endothelium, these interactions
can promote growth of the primary tumour and the establishment of distant
metastatic deposits (Fig. 1.13; Ben-Baruch 2008; Singh et al. 2011).
Figure 1.13 The role of chemokines and their receptors in tumour metastasis.
Chemokines act at various levels within the tumour microenvironment to encourage tumour growth, angiogenesis, invasion and metastasis. Within the tumour mass, tumour cells and infiltrating immune cells both release pro-inflammatory cytokines which can stimulate the production of chemokines. As a result, chemokine receptors can be up-regulated on the surface of tumour cells to promote tumour cell adhesion via integrins, and to enhance cell invasion through the activity of matrix metalloproteinases (MMPs) (Busillo et al. 2007). The tumour cells displaying enhanced expression of chemokine receptors are then equipped to migrate towards microenvironments in which their ligands are expressed. After entry into local blood vessels through interactions with the endothelium via the process of intravasation, tumour cells exit at distant sites via extravasation. At the metastatic site, growth factors and chemokines are expressed within the tissues; if this environment is favourable for tumour growth, metastatic deposits can develop. Figure adapted from O’Hayre et al (2008).

The progression from normal prostate epithelial tissue to a malignant state is accompanied by changes in the expression of chemokines and their receptors. PCa tumours express the CC chemokine receptors CCR6 and CCR5, which are linked with stimulation of cell growth and invasion (Vaday et al. 2006; Vindrieux et al. 2009). PCa tissue displays elevated expression of CXC chemokine receptors CXCR1, CXCR4 and CXCR2 (Balkwill 2004; Murphy et al. 2005). In addition to
regulation of tumour growth, both CC and CXC chemokines can regulate immune cell infiltration of the tumour mass (Kulbe et al. 2004). The presence of immune cells such as leukocytes, lymphocytes and macrophages in the tumour environment provides another source of chemokines and growth factors for tumour cells. However, there is conflicting data as to whether immune cell infiltration encourages disease progression (Ben-Baruch 2008; Kulbe et al. 2004).

Other chemokine receptors can exert a level of control over tumour development. An in vivo study has indicated that the CXC chemokine receptors CXCR2 and CXCR3 may have opposing effects during prostate tumour development in mice (Shen et al. 2006). The effect of CXCR2 or CXCR3 inhibition was investigated in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, which spontaneously develop prostate tumours (Shen et al. 2006). Inhibition of CXCR3 produced larger and more vascularised tumours, whilst CXCR2 inhibition resulted in smaller and less vascularised tumours (Shen et al. 2006).

The ability of tumour cells to move through the endothelium and establish metastatic deposits is largely regulated by their expression of adhesion receptors and the presence of particular chemoattractants in the destination microenvironment (Fig. 1.13; Ben-Baruch 2008). The ‘seed and soil’ hypothesis, originally proposed by Stephen Paget, dictates that different organs (soils) have different microenvironments to which particular tumour cells (seeds) are attracted to (Fidler 2002). In this respect, chemokines and their receptors play a significant role in what is termed the ‘organ selectivity’ of cancer metastasis (Ben-Baruch 2008).

Specifically, target sites express particular chemoattractant chemokines which act to stimulate the directional migration of cancer cells to new microenvironments (Ben-Baruch 2008). In addition to their role in chemoattraction, chemokine receptors can promote tumour cell-endothelial interactions in the process of transendothelial migration, and stimulate integrin and matrix metalloproteinase (MMP) activity to facilitate tumour cell adhesion and invasion (Kulbe et al. 2004).
Particular chemokines known to play a role in mediating PCa metastasis are IL-8 (also known as CXCL8) and stromal-derived factor 1-alpha (SDF-1α, also known as CXCL12) (Kim et al. 2001; Mochizuki et al. 2004; Vindrieux et al. 2009; Wang et al. 2006). Higher levels of IL-8 expression were observed in prostate tumours with higher Gleason scores, and in patients with bone metastasis versus localised PCa. Higher IL-8 observed in patients during PCa progression and when bone metastases are evident (Vindrieux et al. 2009). Studies in vitro have demonstrated IL-8 to promote cell growth, invasion and the production of pro-angiogenic vascular endothelial growth factor (VEGF) and MMP-9 (Vindrieux et al. 2009). When studied in mouse xenograft models, IL-8 over-expression in PCa cells promoted tumour growth and vascularisation, and increased the frequency of lymph node metastasis (Kim et al. 2001). The expression of SDF-1α in the bone marrow and lymph nodes has been established to promote the metastasis of PCa tumour cells. Originally characterised in BCa, PCa tumour cells also displayed over-expression of the SDF-1α receptor CXCR4, and preferentially established metastatic deposits at the bone marrow and lymph nodes of mice (Muller et al. 2001). In vitro studies have demonstrated that the SDF-1α/CXCR4 axis functions to induce PCa cell adhesion and transendothelial migration, which are the primary mechanism used by PCa cells to spread to bone (Kukreja et al. 2005; Taichman et al. 2002).

1.5.3 CXCR4

CXCR4 is commonly expressed on leukocytes, DCs, epithelial cells and haematopoietic progenitor cells, and facilitates cell proliferation, survival, and the homing of cells within the bone marrow (Bleul et al. 1997 Balkwill 2004). CXCR4 contributes towards haematopoiesis, organogenesis and tissue vascularisation, in addition to regulation of embryonic neuronal differentiation and migration (Nagasawa et al. 1998; Stumm et al. 2003). The central role of CXCR4 in these developmental processes was emphasised by the finding that CXCR4 knockout mice die in early development due to defects in haematopoietic, circulatory, central nervous and immune systems (Nagasawa et al. 1996). In the adult, CXCR4 regulates the homing of haematopoietic cells to the bone marrow, and the migration of immune cells to sites of tissue damage as part of the inflammatory response (Balkwill 2004).
CXCR4 is known to bind to one ligand, SDF-1α. Mice deficient in SDF-1α die during development, due to major problems with cardiac septum and bone marrow development, as do CXCR4 knockout mice (Wang et al. 2006). SDF-1α is secreted by bone marrow stromal cells (osteoblasts), endothelial cells, heart and skeletal muscle, liver, brain, kidney and parenchymal cells. Expression of SDF-1α is enhanced during tissue ischemia, excessive bleeding, and exposure to various radiation agents. The release of SDF-1α in damaged tissues promotes the recruitment of stem cells, to initiate tissue regeneration and repair (Wang et al. 2006). SDF-1α can also bind to another chemokine receptor, CXCR7 (Burns et al. 2006). However, despite their overlapping ligand specificities, the functions of these two receptors do not appear to be completely redundant. The in vivo perinatal mortality observed in CXCR4 knockout mice was not rescued by CXCR7 function, indicating that these receptors can mediate distinct pathways during development (Burns et al. 2006).

1.5.3.1 Ligand-Induced CXCR4 Signalling

The binding of SDF-1α to CXCR4 triggers a cascade of signalling events that culminate in the functional cell migration response. After ligand binding, the intracellular effects of CXCR4 activation are mediated through an association with β-arrestins and G proteins. β-arrestins directly interact with CXCR4 as signalling scaffolds upon which intracellular signalling events can occur (Fig. 1.14; Busillo et al. 2007). CXCR4 couples to the G_i family of proteins, inhibiting adenylyl cyclase activity and activating PLC via Src kinase (Busillo et al. 2007). Subsequent activation of the PI3K and MAPK pathways leads to gene transcription, cell adhesion and migration (Busillo et al. 2007; Sun et al. 2010b). Termination of the CXCR4 signalling response can occur through β-arrestin-mediated uncoupling from G proteins, and internalisation of the receptor via endocytosis in clathrin-coated vesicles (Signoret et al. 1997). Internalised CXCR4 can be ubiquitinylated and degraded, or alternatively, recycled back to cell surface to respond to further ligand stimulation (Busillo et al. 2007).
CXCR4 is a seven-transmembrane G protein-coupled receptor that resides in the cell membrane. The extracellular portion of the receptor binds to its ligand, SDF-1α. Activation of CXCR4 induces the association of G proteins on the intracellular portion of the receptor, triggering an interaction with β-arrestin. Downstream intracellular signalling pathways are then activated, resulting in the functional responses. Figure adapted from Woodard et al (2011).

CXCR4 can also activate intracellular signalling in a G protein-independent manner, to stimulate the janus activated kinase-signal transducer and activator of transcription (JAK-STAT) pathway, among others (Vila-Coro et al. 1999). Receptor oligomerisation and co-translational modifications such as N-linked glycosylation can exert a further level of regulation on CXCR4 activity (Busillo et al. 2007).

1.5.3.2 CXCR4 in Cancer

CXCR4 is the most commonly over-expressed chemokine receptor in human cancers, being elevated in over 23 different cancers including those of epithelial, mesenchymal and haematopoietic origin (Balkwill 2004; Wang et al. 2006). This is in contrast to normal tissues such as the breast, ovary and prostate, in which there is no detectable CXCR4 expression. The expression of its ligand, SDF-1α, is constitutive in the lymph nodes, lung, liver and bone marrow: Organs to which cancer cells tend to metastasise. These include BCa, small cell lung cancer, thyroid, neuroblastoma, haematological and hepatic malignancies (Wang et al. 2006). These reports have provided a strong base of evidence for the central role of the SDF-1α/CXCR4 pathway in cancer metastasis.

The role of the SDF-1α/CXCR4 axis in cancer metastasis to the bone marrow was first characterised in BCa (Muller et al. 2001). In addition, the expression of
numeros chemokine receptors is modified in BCa cells when compared to normal breast epithelial cells; the most dramatic changes being an increase in the mRNA expression of CXCR4 and CCR7 (Muller et al. 2001). Inhibition of CXCR4 in vivo resulted in a significant reduction in lymph node and lung metastasis in mice (Muller et al. 2001).

It was based on this research that the pivotal role of CXCR4 was established in the metastasis of another steroid-hormone driven disease, PCa. Interestingly, both of these cancers appear to have very similar patterns of reliance on the SDF-1α/CXCR4 axis for disease progression. Initial reports showed that CXCR4 mRNA and protein was expressed in PCa cell lines, and was absent in normal prostate epithelial cells (Mochizuki et al. 2004). In vivo, over-expression of CXCR4 in mice significantly increases the extent of bone metastasis (Wang et al. 2006). Histological staining of primary tumour tissue samples have shown that, while normal prostate tissue expresses very little or no CXCR4, it was detected in 94.2% of patients with bone metastasis. Higher CXCR4 expression correlated with poorer cancer-specific survival (Akashi et al. 2008). Further research suggests that CXCR4 has the potential to be used as a prognostic marker for bone metastasis, whereby patients with positive CXCR4 are 4.5 times more likely to have bone metastasis (Mochizuki et al. 2004).

More recent studies have shown that CXCR4 may aid in maintenance of PCa progenitor cells or stem-like cancer cells (Dubrovská et al. 2012). PCa stem cells are defined as highly tumorigenic and self-renewing populations which may contribute towards tumour development, and the recurrence of the disease after cancer treatment (Dalerba et al. 2007). Enhanced CXCR4 expression was observed in stem-like cancer cell populations of DU145 and PC3 cells, defined by their expression of stem cell markers CD44, CD133 and integrin α2β1 (Dubrovská et al. 2012). When investigating the functional role of CXCR4 in this context, it was shown that this receptor can influence self-renewal, differentiation potential, cell adhesion, and the tumorigenicity of these stem-like populations (Dubrovská et al. 2012). Based on these findings, the use of anti-CXCR4 therapies may be an effective approach to combat the recurrence of PCa after treatment (Dubrovská et al. 2012).
The actions of SDF-1α on PCa cell lines include cell migration, transendothelial migration, adhesion to endothelial cells and the ECM, particularly through modulation of integrin expression (Chinni et al. 2006). Treatment with SDF-1α activates MMP-9 functions and the release of pro-angiogenic factors VEGF and IL-8 (Chinni et al. 2006). SDF-1α stimulation can also promote up-regulation of αvβ3 integrin to simulate adhesion and invasion, in both PC3 cells and the metastatic LNCaP subline, C4-2B (Wang et al. 2006). SDF-1α can also increase the expression of CXCR4 in PC3 cells, due to activation of the transcription factor NF-κβ downstream of MAPK signalling (Wang et al. 2006). Endogenous SDF-1α acts to stimulate cell proliferation in metastatic PCa cell lines and tumour cells (Mochizuki et al. 2004; Taichman et al. 2002). SDF-1α also stimulates the production of pro-angiogenic cytokines in a cell-type dependent manner. In PC3 cells, SDF-1α-induced PI3K/Akt activation results in production of tissue inhibitor of metalloproteinase 2 (TIMP-2) and VEGF, whereas in LNCaP cells the SDF-1α-induced activation of MAPK or ERK kinase (MEK)/ERK pathway stimulates IL-6 and IL-8 production (Wang et al. 2005a). Increased levels of SDF-1α in tumours may promote angiogenesis and growth of the malignant mass (Duda et al. 2011). Clinical investigations and preclinical studies in solid tumours have demonstrated SDF-1α to be elevated in response to cytotoxic (Kozin et al. 2010; Redjal et al. 2006), vascular disrupting (Shaked et al. 2008), and radiation-based cancer treatments (Chang et al. 2009). As such, SDF-1α may contribute to resistance of tumour cells to such therapies (Duda et al. 2011).

Collectively, these studies have demonstrated that the SDF-1α/CXCR4 axis contributes to the metastasis of BCa and PCa (Fig. 1.15). However, this must be considered in combination with many other factors present in the tumour microenvironment, which also determine the final metastatic spread (Ben-Baruch 2008). Further studies are now required to characterise how the SDF-1α/CXCR4 axis mediates cross-talk with other pro-invasive pathways as part of the tumour cell dissemination process of cancer metastasis.
Malignant cells within the primary tumour that express chemokine receptors such as CXCR4 are better equipped to migrate away from the primary mass. The production of inflammatory cytokines, MMPs and SDF-1α stimulate cell migration and invasion, resulting in intravasation of tumour cells into blood vessels. Once in the circulation, the tumour cells travel to and exit at distant sites. In particular, tumour cells expressing high levels of CXCR4 tend to spread to microenvironments that express high levels of SDF-1α – the bone marrow and lymph nodes in particular. Metastatic deposits develop at these sites, resulting in the formation of clinically detectable metastases. Figure adapted from Sun et al (2010b).
1.5.3.3 Regulation of CXCR4

*In vitro* studies of BCa have demonstrated that activity of the oestrogen receptor (ER) enhances the expression of SDF-1α and CXCR4 (*Hall et al.* 2003). ER can increase CXCR4 expression by activating NF-κB, which is able to bind directly to the CXCR4 promoter to initiate gene transcription (*Helbig et al.* 2003). CXCR4 can also be regulated post-transcriptionally, by cross-talk between Her2 and ER via the PI3K/Akt, MAPK and mammalian target of rapamycin (mTOR) pathways (*Sengupta et al.* 2008). Due to its regulation of CXCR4, ER activity can also up-regulate SDF-1α production, which can stimulate cell proliferation (*Hall et al.* 2003).

CXCR4 expression is also regulated by AR in PCa cell lines *in vitro*. Transfection of the DU145 PCa cell line with AR can negatively influence CXCR4 mRNA expression and SDF-1α-induced cell migration in the DU145 cell line (*Akashi 2006*). These findings converge with altered integrin and pro-angiogenic cytokine expression profiles, and the inhibition of SDF-1α-induced migration by androgens (*Nagakawa 2004; Nagakawa 2002*). *Akashi* and colleagues (2006) also demonstrated an inhibition of CCR1 mRNA expression and the CCL3/MIP-1α-mediated migration response, showing that the effect is not unique to CXCR4. However, in comparison, the influential role of CXCR4 in PCa bone metastasis has warranted special interest.

More recently, studies have demonstrated that this regulation of CXCR4 can extend to androgen-sensitive PCa cell lines. Treatment of LNCaP cells with R1881 synthetic androgen increased the level of both CXCR4 mRNA and functional CXCR4 protein (*Frigo 2009*). Specifically, stimulation of AR with R1881 activated the CXCR4 transcription factor Krüppel-like Factor 5 (KLF5). As a consequence, CXCR4-mediated cell migration was enhanced in the presence of SDF-1α (*Frigo 2009*).

Another group utilising microarray studies have demonstrated that treatment of LNCaP cells with either R1881 synthetic androgen or DHT produced a 20- or 1.9-2.5-fold increase in CXCR4 mRNA levels, respectively (*Ngan 2009*). Finally, studies investigating the androgen-sensitive VCaP cell line have linked the androgen regulation of CXCR4 with expression of the avian v-ets erythroblastosis virus E26 oncogene homolog (ERG) (*Cai 2010*). The ERG oncogene can combine
with the AR target gene transmembrane protease serine 2 (TMPRSS2) to produce a fusion protein (TMPRSS2-ERG) that is found in up to 50% of sampled PCa tumours (Park 2010b). Over-expression of TMPRSS2-ERG has been associated with tumour stage, and is linked with expression of pro-proliferative genes in PCa (Lehmusvaara et al. 2012; Pettersson et al. 2012). Interestingly, treatment of VCaP cells with R1881 can induce binding of TMPRSS2-ERG to the CXCR4 promoter and enhance expression of the CXCR4 protein (Cai 2010).

Evidence of a mutual regulation between CXCR4 and AR was demonstrated by a more recent study in LNCaP cells (Kasina 2012). SDF-1α treatment resulted in activation of AR in the absence of androgens, as reflected by nuclear translocation of AR, enhanced AR-dependent cell proliferation, and the transcription of the AR target gene, PSA (Kasina 2012). As such, it is evident that SDF-1α can act as a non-steroidal growth factor to promote the AR-dependent growth of prostate epithelial cells in the absence of androgens.

Collectively, this evidence demonstrates a connection between CXCR4, androgens and PCa progression. However, CXCR4 expression and activity has recently been linked with CXC chemokine receptor 7 (CXCR7) (Boudot et al. 2011; Singh et al. 2011; Wang 2008). Thus, it is unlikely that the SDF-1α/CXCR4 axis is acting independently of the more recently discovered CXCR7 pathway in PCa.

1.5.4 CXCR7

The expression of the alternative SDF-1α-binding receptor, CXCR7, has also been found to correlate with PCa progression. Studies in vitro have found that this receptor can regulate tumour cell growth, apoptosis and stimulate PCa cell invasion (Wang 2008). The initial characterisation of CXCR7 as an SDF-1α binding receptor in cancer was performed in BCa cells (Burns et al. 2006). Prior to this study, CXCR4 and SDF-1α were considered to be a monogamous ligand-receptor pair, and all SDF-1α mediated responses were attributed to CXCR4 activity (Burger et al. 2006).

In BCa cell lines, disparities were observed between SDF-1α uptake and CXCR4 expression (Burns et al. 2006). In addition, murine cells derived from CXCR4 knockout embryos were capable of binding radio-labelled SDF-1α, in a manner
that was not inhibited by the CXCR4 antagonist, AMD3100 (Burns et al. 2006). The receptor was identified as the orphan receptor RDC1. The function of this second SDF-1α binding receptor was investigated through transfection of the RDC1 sequence into the CXCR4-negative, MDA-MB-438 BCa cell line (Burns et al. 2006). High-affinity SDF-1α-binding was observed to be displaced by another ligand, interferon-inducible T cell alpha chemoattractant (ITAC, or CXCL11). Based on its sequence and chemokine-binding properties, RDC1 was then classified as a seven-transmembrane receptor of the chemokine receptor family, and named CXCR7 (Burns et al. 2006).

1.5.4.1 Characterisation of CXCR7

Previously known as the orphan receptor RDC1, CXCR7 was originally cloned from a dog thyroid cDNA library using PCR primers which amplify transmembrane domains of known GPCRs (Libert et al. 1990). The RDC1 gene displayed 90% homology between dog, human and mouse (Heesen et al. 1998). The efforts of Burns and colleagues (2006) in characterising this receptor resulted in identification of its binding activity with the ligands SDF-1α and ITAC, and the classification of this receptor within the CXC chemokine receptor family based on the structure of its amino acid sequence. Although it binds to both chemokines, this receptor has a 10-fold lower affinity for ITAC when compared to SDF-1α; ITAC also being a ligand for the closely related receptor CXCR3, and SDF-1α a ligand also for CXCR4 as described previously (Burns et al. 2006).

CXCR7 is considered an atypical member of the chemokine family. Subsequent to ligand binding, CXCR7 does not mediate the typical cell chemotaxis or calcium mobilisation response of chemokine receptors (Burns et al. 2006). Rather than directly coupling intracellularly to G proteins, CXCR7 preferentially associates and signals through β-arrestin 2 to mediate chemokine uptake via receptor internalisation (Décaillot et al. 2011; Ray et al. 2012; Thelen 2001). The internalisation of CXCR7 via clathrin-mediated endocytosis directs the receptor through early endosomes, late endosomes, and lysosomes (Ray et al. 2012). In this manner, CXCR7 can efficiently act as a chemokine scavenger by internalising extracellular ligands for degradation, prior to recycling of the receptor back to the cell surface (Ray et al. 2012).
Activation of MAPK and PI3K/Akt signalling pathways have been reported downstream of ligand-induced CXCR7 activation (Fig. 1.16; Ray et al. 2012; Wang 2008). The carboxy-terminus of this receptor is particularly important for multiple functions of the receptor, including association with β-arrestin 2, chemokine uptake and ligand-dependent activation of ERK 1/2 (Ray et al. 2012).

![CXCR7 activation after ligand binding](image)

**Figure 1.16 CXCR7 activation after ligand binding.**
The binding of CXCR7 by its chemokine ligands (either SDF-1α or ITAC) results in receptor activation, after which it signals through β arrestin 2. This allows for receptor internalisation and ligand degradation, and can elicit activation of the MAPK and PI3K/Akt signalling pathways. Figure adapted from O’Hayre et al. (2008).

### 1.5.4.2 The Functions of CXCR7

CXCR7 expression has been observed in various cell types during embryonic development, and on neuronal, endothelial cells and osteocytes in the adult (Burns et al. 2006; Gerrits et al. 2008; Schonemeier et al. 2008; Sierro et al. 2007). The role of CXCR7 appears to differ markedly between different cell types; in some cases it can act as a non-signalling decoy receptor (human embryonic kidney 293 cells) or as an SDF-1α scavenger (madin-darby canine kidney epithelial cells) to regulate levels of extracellular SDF-1α and ITAC (Dambly-Chaudiere et al. 2007; Naumann et al. 2010; Rajagopal et al. 2010). Along with CXCR4 and CCR5, CXCR7 was also identified as a co-receptor for specific strains of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) (Shimizu et al. 2000).
CXCR7 has been shown to play an active role in developmental processes. Research in zebrafish models *in vivo* has demonstrated that CXCR7 is required, along with CXCR4, for directional cell migration patterns during organ morphogenesis in embryonic development (Dambly-Chaudiere *et al.* 2007; Valentin *et al.* 2007). Indeed, CXCR7 functionality is required during cardiac development in murine models, and is essential for perinatal survival (Gerrits *et al.* 2008; Sierro *et al.* 2007; Yu *et al.* 2011). CXCR7 knockout mice displayed thickening of aortic and pulmonary valves, resulting in severe cardiac defects and death, due to insufficient blood supply to peripheral tissues (Sierro *et al.* 2007; Yu *et al.* 2011). Another study revealed that CXCR7 knockout mice displayed cardiac hypertrophy due to hyperplasia, resulting in lethality (Gerrits *et al.* 2008).

CXCR7 is also expressed in the developing and adult rat brain and may play a role in the central nervous system (CNS) (Schonemeier *et al.* 2008). A recent study showed that CXCR7 is required for migration of cortical interneurons, and positioning within the cortical plate during CNS development in mice (Wang *et al.* 2012). Although CXCR7 is expressed widely across various adult tissues at the mRNA level, its protein expression at the cell membrane is much more restricted, perhaps due to post-translational regulation (Burns *et al.* 2006). Differentiated neurons in the adult brain retain CXCR7 expression (Shimizu 2011), as well as immune cells such as T lymphocytes, DCs and B cells (Balabanian *et al.* 2005).

### 1.5.4.3 CXCR7 in Cancer

Although its expression is highly restricted, CXCR7 expression is closely associated with malignant tissue in the adult, displaying high levels of expression in tumours of the breast, prostate, lung, liver and colon (Burns *et al.* 2006; Duda *et al.* 2011; Maio 2007; Monnier 2012; Wang 2008). The level of CXCR7 expression can be associated with poor survival in gallbladder cancer, cervical cancer and disease progression in bladder cancer (Hao *et al.* 2012). Expression of CXCR7 was also observed to correlate with tumour grade and invasive stage of both hepatocellular carcinoma and pancreatic adenocarcinomas (Gebauer *et al.* 2011; Monnier 2012; Zheng *et al.* 2010).

CXCR7 can promote tumour development by regulating the growth and survival of tumour cells (Burns *et al.* 2006; Maio 2007; Wang 2008).
CXCR7 is associated with enhanced growth of cell lines in vitro, including those derived from breast, prostate and lung cancers (Burns et al. 2006; Maio 2007; Monnier 2012; Wang 2008; Xu 2011). In particular, CXCR7 expression can support BCa cell growth under suboptimal conditions in vitro, by enhancing cell survival (Burns et al. 2006). CXCR7 can also promote tumour growth through its expression on tumour-associated blood vessels, stimulating the vascularisation process (Burns et al. 2006; Maio 2007; Wang 2008). A central role of CXCR7 in BCa tumour development was demonstrated in mice in vivo (Burns et al. 2006). Administration of a CXCR7 antagonist in murine models of BCa provided a 50% reduction in the incidence of tumours (Burns et al. 2006). The tumours that did develop were poorly organised and vascularised, and not encapsulated (Burns et al. 2006).

CXCR7 has also been demonstrated to influence promote tumour growth, angiogenesis and invasion in PCa (Fig. 1.17; Wang 2008). Higher CXCR7 expression is associated with increased tumour grade in PCa biopsy samples, and knockdown of this receptor in murine models significantly decreased tumour volume (Wang 2008). Studies in vitro have shown CXCR7 expression in PCa cells permits enhanced cell survival, adhesion and invasion (Wang 2008). Cell cycle arrest was induced in both normal prostate epithelial and PCa cell lines after inhibition of CXCR7 using an siRNA approach in vitro (Singh et al. 2011). Another mechanism by which CXCR7 may promote tumour growth is mediating the expression of pro-angiogenic VEGF and IL-8 via the Akt signalling pathway (Singh et al. 2011; Wang 2008).

**Figure 1.17** Effects mediated by CXCR7 in PCa cells.
The expression of CXCR7 on PCa cells results in enhanced cell survival, cell adhesion to endothelial cells, cell invasion and increased production of pro-angiogenic cytokines IL-8 and VEGF (Wang et al. 2008a)
1.5.5 Regulation of CXCR7 and CXCR4

The combined roles of SDF-1α, CXCR4 and CXCR7 in regulating tumour cell growth, survival, invasion and angiogenesis have revealed this axis to be a therapeutic target in PCa (Duda et al. 2011; Sun et al. 2010a; Woodard 2011). In addition to their shared ligand specificity, the expression of CXCR7 and CXCR4 can display an inverse regulation in vitro. In PCa cell lines, CXCR7 expression is negatively regulated by CXCR4 over-expression (Wang et al. 2008a). The growth and proliferation of PCa cells is enhanced by IL-8-mediated up-regulation of CXCR7, coinciding with a down-regulation of CXCR4 expression (Singh et al. 2011). In BCa cell lines, oestrogen was observed to repress CXCR7 expression whilst up-regulating CXCR4 and SDF-1α to promote cell growth (Boudot et al. 2011). In glioma cells, an inverse regulation was also observed between CXCR7 and CXCR4 expression levels (Hattermann et al. 2012). CXCR4 expression was higher in glioma stem-like cells, and expression levels decreased as the cells differentiated. During differentiation, CXCR7 expression was increased, promoting cell survival (Hattermann et al. 2012).

CXCR7 is known to occur as a homodimer, or a heterodimer with CXCR4 (Levoye et al. 2009; Luker et al. 2009; Sierro et al. 2007; Wang et al. 2006). When both receptors are transiently over-expressed in vitro, formation of the CXCR4:CXCR7 heterodimer recruits β-arrestin and elicits downstream signalling pathways (Décaillot et al. 2011). Consequently, some actions of CXCR7 can be dependent on its ability to cooperate with CXCR4, whilst others can occur independently of this association (Fig. 1.18). Heterodimerisation between these receptors can modulate SDF-1α-induced tumour cell migration and transendothelial migration in vitro (Zabel 2009). It is therefore evident that CXCR7 can elicit responses either independently or in combination with CXCR4.
CXCR7 can elicit function responses to SDF-1α both independently of, and in combination with, CXCR4.

The binding of ligand to CXCR4 leads to the association of G proteins. In turn, the MAPK and PI3K/Akt intracellular signalling pathways are activated, leading to enhanced cell survival, proliferation and chemotaxis (cell migration). Alternatively, the binding of SDF-1α to CXCR7 results in internalisation and degradation of SDF-1α. Formation of the CXCR7-CXCR4 heterodimer leads to stimulation of cell survival, proliferation and cell migration. Figure adapted from Duda *et al.* (2011).

### 1.5.6 Pharmacological Intervention of the Chemokine Network in Cancer

Currently, there are very limited treatment options available for patients with advanced PCa, particularly in those who have shown progression to metastasis. There is a need for alternative treatments; in particular, therapies to inhibit dissemination of tumour cells from the prostate. Many members of the chemokine network regulate tumour formation and cancer progression by assisting in tumour growth, invasion and metastasis. This network is a potential target for future cancer therapies, with the potential to inhibit tumour growth and/or restrict dissemination of cancer cells from the primary site (Duda *et al.* 2011; Singh *et al.* 2010; Vandercappellen *et al.* 2008). Successful outcomes may include improved quality of life and delayed progression to mortality (Singh *et al.* 2010). This delayed disease progression may allow more effective utilisation of current anti-cancer therapies including chemotherapy and radiotherapy to control tumour growth (Singh *et al.* 2010).

The concept of targeting the chemokine network for inhibition is, however, accompanied by a variety of complications (Ben-Baruch 2008; Vandercappellen *et al.* 2008). The chemokines and their receptors are highly redundant, and their biological role and functionality are overlapping and complex. The overlapping
specificity of chemokines for chemokine receptors and the redundancy of their biological roles may lead to compensatory mechanisms emerging, potentially resulting in low efficacy of anti-chemokine or anti-chemokine receptor drugs (Ben-Baruch 2008). For example, SDF-1α binds to both CXCR4 and CXCR7; however it is unclear whether, and to what extent, CXCR7 may be affected when CXCR4 functionality is inhibited (Ben-Baruch 2008). Secondly, the roles of chemokines are multifaceted and can change under different circumstances and conditions. Some can encourage or inhibit tumorigenesis and metastasis under different circumstances (Ben-Baruch 2008). Therefore, targeting a member of the chemokine receptor family for inhibition of cancer progression may have opposing effects dependent on the stage of disease.

The finding that CXCR4 antagonists can stimulate CXCR7 shows that selective inhibition may not be simple (Maksym et al. 2009; Sun et al. 2010a). Also, SDF-1α acts through both CXCR4 and CXCR7, and accordingly, blockage of only one of these receptors may not be an effective approach to inhibition of SDF-1α activity (Maksym et al. 2009; Sun et al. 2010a). Combination therapies targeted at both CXCR4 and CXCR7 may be effective in this respect, or alternatively, targeting common downstream pathways of both receptors for inhibition (Fig. 1.19; Maksym et al. 2009; Sun et al. 2010a). Such therapies could be employed to enhance the effectiveness of currently available treatments, through their administration as adjuvant therapies (Duda et al. 2011; Woodard 2011).
Figure 1.19 Targeting the CXCR4/CXCR7 axis for inhibition in PCa.
Potential therapeutic benefit may be obtained from inhibition of the CXCR4/CXCR7 axis in PCa, including the inhibition of tumour growth, invasion and angiogenesis. The first potential point of intervention is the ligand-receptor binding interaction. Inhibition of this would prevent receptor activation and subsequent downstream activities. A second point of intervention is the activation of shared signalling pathways between these receptors. Inhibition of this response may prevent gene transcription and the associated effects on cell adhesion, invasion, growth and angiogenesis (Maksym et al. 2009; Sun et al. 2010a).

Issues that may complicate the use of anti-chemokine receptor therapies in the clinic include non-redundant functions required for biological homeostasis. The maintenance of immune populations in particular organs, the turnover of tissue in the adult, and the migration of newly generated populations may require the non-redundant functions of these receptors (Ben-Baruch 2008). SDF-1α and CXCR4 are essential for in vivo development and haematopoiesis, and consequently, blocking functionality of this axis may have undesired side effects that cannot be compensated for (Ben-Baruch 2008; Vandercappellen et al. 2008). Hence, the side effects of anti-chemokine receptor therapies need to be examined closely, particularly regarding long-term administration. However, it has been established that short-term treatment with CXCR4 antagonist AMD070 is safe in human trials of healthy males, when administered at 12 hour intervals for up to 84 hours (Sung et al. 2007). The potential use of CXCR4 inhibitors for cancer treatment may be in their potential utility as a short-term adjuvant therapy, combined with other currently available treatments (Woodard 2011).
However, some effects of these inhibitors can be beneficial, expanding the potential clinical applications of chemokine receptor antagonists. Clinical trials of the CXCR4 antagonist AMD3100 (also known as Plerixafor or Mozobil®) have demonstrated that antagonism of CXCR4 is accompanied by an increase in white blood cell counts in peripheral blood (De Clercq 2009). The ability of AMD3100 to mobilise stem cells from the bone marrow has made it highly effective in stem cell mobilisation therapy for patients with haematological malignancies (De Clercq 2009; Keating 2011). Administration of this drug prevents the SDF-1α/CXCR4-mediated retention of stem cells in the bone marrow, allowing the movement of these cell populations into the circulation (De Clercq 2009). Re-stimulation of haematopoietic stem cell function is required after chemotherapy or radiation in patients with non-Hodgkins lymphoma, leukemia or myeloma. Treatment with granulocyte colony stimulating factor (GCSF) can elicit the required stem cell mobilisation response, a process which can be enhanced through co-administration of AMD3100 (De Clercq 2009; Keating 2011). Clinical studies have shown this method to be highly effective and well tolerated (Keating 2011).

There are currently no specific inhibitors of CXCR4 or CXCR7 available in the clinic. AMD3100 was originally thought to bind specifically to CXCR4, but was later found to also bind to CXCR7 and stimulate receptor activation (Gravel et al. 2010; Kalatskaya et al. 2009). Due to the redundancy within the chemokine network, dual-targeted or combination therapies may be a more feasible option for targeting the metastatic process for inhibition (Fig. 1.19). To achieve this end, further investigation regarding the regulation of the chemokine network in cancer is required. Specifically, the distinct functions of CXCR7 and CXCR4 require further delineation, as they are currently unclear. The efforts to further define the specific functions of CXCR7 are hampered by its shared ligand specificity, and high-affinity, CXCR7-specific small molecule agonists are under development for this purpose (Wijtmans et al. 2012). It is essential to further characterise the redundancy in the chemokine network, in order to understand the complexity, implications and limitations of their therapeutic potential. Knowing more about regulation between central pathways in PCa may contribute towards a better
understanding of how to circumvent, or even utilise, this redundancy in cancer biology.
1.6 Justification of Research

CXCR4 plays a major role in the processes of migration, invasion and angiogenesis of tumours as part of the PCa development and progression. Targeting this chemokine receptor alone, however, is complicated by its relationship with CXCR7. To more effectively combat PCa progression, a more detailed knowledge of chemokine regulation mechanisms is required.

To further decipher the role of CXCR4 in PCa and its association with CXCR7, the mechanisms regulating CXCR4 protein expression and function were investigated in PCa cell lines in conjunction with CXCR7. The regulation of CXCR4 by androgens in PCa cell lines were studied in vitro using both conventional 2D culture and the more physiologically relevant 3D cell culture model. The relationship between CXCR4 and CXCR7 was then studied to determine how these receptors are regulated in relation to each other, in the presence of a supportive ECM. Knowing more about regulation of these pathways and how this is affected by the presence of ECM and spheroid morphology may contribute towards elucidating regulatory mechanisms behind the progression of PCa. Further characterisation of CXCR7 function with respect to CXCR4 will shed light on how these receptors contribute to regulation of the metastatic process in PCa – a process known to be heavily regulated by CXCR4. A more detailed knowledge of the CXCR7 receptor, with which CXCR4 shares a partial redundancy, may indicate whether combinatorial or selective therapies may prove to be more effective in combating PCa progression.
1.7 Thesis Aims and Hypotheses

The overall aims of this thesis were to investigate the regulation of CXCR4 by androgens and the ECM in PCa cells, and to determine the consequences on functional behaviours that are associated with tumour cell dissemination.

As a basis for studies into regulation of CXCR4 by androgens, Chapter 3 involved investigation into the endogenous protein expression of AR in 2D and 3D culture. The hypothesis of that chapter was that culturing PCa cell lines in 3D would result in altered AR protein expression. The protein expression of AR was compared between 2D and 3D culture, using Western blot, immunocytochemistry and script-based image analysis.

The aim of Chapter 4 was to investigate the regulation of CXCR4 protein expression and function in 2D and 3D culture; using Western blot, immunocytochemistry and script-based image analysis. It was hypothesised that the culturing of PCa cell lines in 3D would result in altered CXCR4 protein expression. Based on those results, the effect of androgen treatment on CXCR4 was investigated. The hypothesis underlying that investigation was that the regulation of CXCR4 by androgens would be observed in both androgen-sensitive and androgen-insensitive PCa cell lines. The study was carried out with the aim to characterise the effect of androgen treatment on CXCR4 protein expression and function in PCa cell lines, in both 2D and 3D culture.

The aim of Chapter 5 was to generate androgen-insensitive PCa stably expressing AR, to use in studies of CXCR4 regulation. The hypothesis behind that study was that regulation of CXCR4 by androgens would be restricted to AR-positive PCa cells. To that end, androgen-insensitive PCa cells were transfected with AR, in order to study the effect on CXCR4 protein expression and function in that system.

Finally, a further level of CXCR4 regulation in relation to CXCR7 protein expression was studied with respect to the surrounding ECM in Chapter 6. The hypotheses of that study was that CXCR7 protein expression would be associated with cell invasive capacity, and that any changes to CXCR4 protein expression in 3D culture will be accompanied by an inverse regulation of CXCR7 protein expression. To investigate that regulation, the protein expression and function of
CXCR7 were studied in 2D and 3D cultures of PCa cell lines using Western blot, immunocytochemistry and script-based image analysis.
2 Chapter Two: Materials and Methods
2.1 General Tissue Culture

2.1.1 Consumables

Tissue culture flasks were purchased from Sigma-Aldrich (Cat. No. C7231). Plates used for cell-based assays were 12-well (Cat. No. CLS3512, Sigma Aldrich), 24-well (Cat. No. CLS3527, Sigma Aldrich), 96-well (Cat. No. 6005550, PerkinElmer) or 384-well plates (Cat. No. 6007558, PerkinElmer). All other general consumables and reagents were supplied by Sigma-Aldrich unless otherwise stated.

2.1.2 2D Cell Culture

The PCa cell lines, LNCaP (clone FGC; Cat. No. CRL-1740), PC3 (Cat. No. CRL-1435) and DU145 (Cat. No. HTB-81) were purchased from American Type Culture Collection (ATCC). LNCaP are androgen sensitive and androgen dependent for growth and proliferation, whereas DU145 and PC3 cells are insensitive to androgen stimulation, and are androgen independent regarding their cell growth and proliferation (summarised in Table 2.1). All cells were maintained in complete medium, consisting of phenol red-free RPMI (Cat. No. A10491-01, Life Technologies) with 10% Fetal Bovine Serum (FBS) (Cat. No. 10082147, Life Technologies), at 37°C with 5% CO₂ and 95% humidity. Phenol red was omitted from the routine culturing medium, due to its ability to act as a steroid receptor agonist (Berthois 1986; Welshons 1988). Cells were passaged twice per week and culture medium was replenished every 2-3 days. Upon reaching 85-90% confluency, cells were harvested using Accutase® solution (Cat. No. A6964) and reseeded to the appropriate densities according to cell growth rate. For assays, cells were plated out in complete medium at the indicated densities, and incubated for 16 hours at 37°C, 5% CO₂ and 95% humidity. After 10-12 passages, cultures were discontinued.
Table 2.1 PCa cell lines used for investigations in this research.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Derived from</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>50 year old male with metastatic prostate adenocarcinoma</td>
<td>Left subventricular lymph node</td>
<td>Produces AR and PSA, androgen sensitive growth, relatively indolent biologic behaviour</td>
<td>(Horoszewicz et al. 1980; Horoszewicz et al. 1983; Van Bokhoven et al. 2003)</td>
</tr>
<tr>
<td>DU145</td>
<td>69 year old male with metastatic prostate adenocarcinoma</td>
<td>Brain</td>
<td>Androgen insensitive AR and PSA negative</td>
<td>(Stone et al. 1978; Van Bokhoven et al. 2003)</td>
</tr>
<tr>
<td>PC3</td>
<td>62 year old male with grade IV metastatic prostate adenocarcinoma</td>
<td>Bone</td>
<td>Androgen insensitive Poorly differentiated carcinoma Aggressive behaviour AR and PSA negative</td>
<td>(Kaighn et al. 1979; Van Bokhoven et al. 2003)</td>
</tr>
</tbody>
</table>

2.1.3 3D Cell Culture

Tissue culture plates (either 12-, 96- or 384-well plates) were coated with a layer of 70% (v/v) Matrigel (Growth Factor-Reduced; Cat. No. 356230, BD Biosciences) diluted in cold serum-free RPMI 1640 medium (SFM), and polymerised for 2 hours at 37°C, 5% CO₂ and 95% humidity prior to seeding cells in complete medium directly on top. The volumes of Matrigel and cells densities used for culturing vessels are shown in Table 2.2. Cultures were maintained for up to 11 days, and culture medium was carefully replaced every 2-3 days. Where specified, cells were serum-starved in the presence of indicated treatments prior to assays.
Table 2.2 Reagent volumes and cell densities used for 3D culture.

<table>
<thead>
<tr>
<th>Format</th>
<th>Volume of 70% Matrigel</th>
<th>Volume of Culture Medium</th>
<th>Cell Density per Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-well plate</td>
<td>350 µl</td>
<td>1,000 µl</td>
<td>20,000 - 40,000</td>
</tr>
<tr>
<td>96-well plate</td>
<td>50 µl</td>
<td>100 µl</td>
<td>3,000 - 5,000</td>
</tr>
<tr>
<td>384-well plate</td>
<td>20 µl</td>
<td>50 µl</td>
<td>800 - 1,000</td>
</tr>
</tbody>
</table>

Signalling pathway inhibition experiments in Chapters 3 and 4 were carried out as follows: LNCaP or PC3 cells were cultured in 3D culture in a 12-well plate format for 9 days. The next day, cells were pre-treated for 16 hours in complete medium containing well characterised signalling pathway inhibitors, at concentrations which have previously been reported to be effective in culture (Boulton et al. 1996; Recchia et al. 2009; Sabbota et al. 2010; Zelivianski et al. 2003). Src family kinase inhibitor PP2 (5 µM; Cat. No. 529573, Merck Millipore), its inactive analogue PP3 (5 µM; Cat. No. 529574, Merck Millipore), the mammalian target of rapamycin (MTOR) inhibitor, rapamycin (1 µM, Cat. No. 553210, Merck Millipore), the PI3K/AKT inhibitor wortmannin (20 µM; Cat. No. W1628), the MAPK inhibitor PD98059 (10 µM; Cat. No. P215) or 0.4% (v/v) DMSO vehicle control were applied to cells for 16 hours. Following this, cells were then serum-starved for 30 hours with the indicated inhibitors, in the presence or absence of 10 nM DHT (Cat. No. A8380). Cells were recovered from 3D culture as detailed in 2.1.3.1, and analysed via Western blot.

2.1.3.1 Extraction of Cells from 3D Culture

After the stated length of time in culture, cells were removed from 3D culture using BD cell recovery solution (CRS; Cat. No. 354253, BD Biosciences), as per the manufacturer’s instructions. Culture medium was carefully removed from 12-well plates and cultures were washed twice with 1 ml of cold PBS (Cat. No. 14190144, Life Technologies). Ice-cold CRS was applied to dissolve the Matrigel matrix at a volume of 1 ml per well. Using a wide-bore 1 ml pipette tip, the cells and matrix were transferred into 15 ml tubes and incubated at 4°C on a rocking platform to more completely solubilise the matrix into solution. The duration of this step was approximately 1.5 hours, or until a visible cell pellet was seen to fall out of solution. Cells were then centrifuged at 4°C (10 minutes, 300 x g), washed
2x with ice-cold PBS (7 minutes, 500 x g), and resuspended according to their subsequent analysis method: (A) Replating for growth in 2D culture, or (B) Western blot analysis.

(A) Cell pellets were resuspended in complete medium and cell counts were performed. After dilution to the appropriate cell densities, cells were seeded into 12 well plates; either directly on the tissue culture (TC)-treated surface (2D condition), or on top of a thin layer of 70% (v/v) Matrigel which retained 2D characteristics and phenotype (2D + Mat).

(B) Cell pellets were resuspended in 100-200 µl of radio-immunoprecipitation assay (RIPA) lysis buffer, and were processed as described in Methods section 2.6.1-2.6.3 for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

2.1.4 Charcoal-Stripped FBS (CS-FBS)

For androgen stimulation assays, CS-FBS was used to minimise interference with endogenous steroid hormones present in the FBS. To prepare CS-FBS, the serum was incubated with 5% (v/v) charcoal dextran solution, consisting of 0.14 M NaCl containing 0.5% (w/v) activated charcoal (Cat. No. C9157) with 0.05% (w/v) Dextran T70 (Cat. No. 17-0280-01, Pharmacia). Serum was incubated with this solution for 30 minutes at 55°C, prior to centrifugation at 4,000 x g for 30 minutes and filter sterilisation (Eckert 1982). CS-FBS was used at a concentration of 10% (v/v) in RPMI-1640 for assays.

2.1.5 Measurement of Prostate Specific Antigen (PSA)

Culture supernatants were collected, centrifuged (10 minutes, 500 x g) to remove debris, and analysed for PSA concentration using a PSA ELISA (Cat. No. DKK300, R&D Systems), according to the manufacturer’s instructions. Readings were obtained using absorbance on the KC4 PowerWave™ Microplate Scanning Spectrophotometer (Bio-Tek). The concentration of PSA in each sample was calculated against a standard curve generated with recombinant PSA supplied within the kit.
2.2 Immunocytochemistry and Imaging

2.2.1 Immunocytochemistry

Cells were washed with phosphate buffered saline (PBS; 3 x 5 minutes), and fixed with paraformaldehyde (PFA, 4% (w/v); Cat. No. 158127). For 2D cultures, cells were fixed for 10 minutes, and 3D cultures were fixed for 20 minutes. After further washing (3 x 5 minutes PBS), blocking buffer was applied for 16 hours at 4°C. Blocking buffer was comprised of 2% (w/v) IgG-free BSA (Cat. No. A5611), 0.1% (v/v) Triton X-100 (Cat. No. X100) and 0.05% (v/v) Tween 20 (Cat. No. P1379). Cells were further washed (1 x 5 minutes PBS/0.1% (v/v) Triton X-100; 2 x 5 minutes PBS) and incubated with primary antibodies (Table 2.3) for 16 hours at 4°C in blocking buffer. Cells were washed with PBS (3 x 5 minutes), and incubated with appropriate secondary antibodies; goat anti-mouse Alexa Fluor 488® (Cat. No. A11001), goat anti-rat Alexa Fluor® 594 (Cat. No. A11007), and/or goat anti-rabbit Alexa Fluor® 594 (Cat. No. A11037) antibody (all 5 µg/ml; Life Technologies). Concurrently with incubation of secondary antibodies, nuclear staining was performed using either DRAQ5™ (1/500; Cat. No. DR71000, Biostatus Ltd), 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1/1000; Cat. No. D9542) or Hoechst 33342 (1/1000; Cat. No. 861405). Additional stains utilised for defining cell shape and morphology included filamentous actin labels Texas Red-phalloidin (1/100; Cat. No. T7471) or Alexa Fluor® 488-phalloidin (1/100; Cat. No. A12379, both from Life Technologies) and HCS CellMask™ Blue (1/500; Cat. No. H32720, Life Technologies), and incubated for 4 hours at room temperature (RT). Cells were washed with PBS, and imaged using an Olympus FV1000 Confocal microscope.
Table 2.3 Primary antibodies used for immunocytochemistry studies.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Details</th>
<th>Species</th>
<th>Catalogue Number</th>
<th>Supplier</th>
<th>Concentration Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>Conjugated to Alexa Fluor 488® Monoclonal</td>
<td>Mouse anti-human</td>
<td>sc7305-488</td>
<td>Santa Cruz Biotechnologies</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Monoclonal</td>
<td>Mouse anti-human</td>
<td>MAB172</td>
<td>R&amp;D Systems</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>CXCR7</td>
<td>Monoclonal</td>
<td>Mouse anti-human</td>
<td>K0223-3</td>
<td>MBL International</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>CXCR7*</td>
<td>Polyclonal</td>
<td>Rabbit anti-human</td>
<td>ab38089</td>
<td>Abcam</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Integrin β1</td>
<td>Monoclonal</td>
<td>Mouse anti-human</td>
<td>MAB17783</td>
<td>R&amp;D Systems</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>β-Laminin</td>
<td>Polyclonal</td>
<td>Rat anti-human</td>
<td>ab44941</td>
<td>Abcam</td>
<td>5 µg/ml</td>
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<tr>
<td>MMP-11</td>
<td>Monoclonal</td>
<td>Rabbit anti-human</td>
<td>ab52904</td>
<td>Abcam</td>
<td>5 µg/ml</td>
</tr>
</tbody>
</table>

* Employed in co-labelling studies in conjunction with mouse anti-CXCR4 antibody

2.2.2 Cell Imaging

All live cell images were collected on an Olympus Cell-R microscope, using an Electron Multiplying Charge-Coupled Device (EMCCD) camera fitted with epifluorescence and Differential Inference Contrast (DIC) optics. Images were collected with an Olympus PlanNeo-FLUAR 20/0.8 air objective. During imaging, cell cultures were maintained at 37°C in an incubator chamber with 5% CO₂. Images of 3D spheroids over time were taken of live cells using DIC.

All fixed cells were imaged using either a PerkinElmer Opera™ Quadruple Excitation High Sensitivity confocal plate reader with a PerkinElmer 20/0.75 water objective, or an Olympus FV1000 Confocal microscope, with an Olympus PlanNeo-FLUAR 40/1 oil objective for multiple z-plane acquisition. Z-stacks of 160-180 z-planes with a step size of ~0.4-0.8µm were acquired with Olympus Fluoview software (Version 1.7b, Olympus). Three-dimensional reconstructions
of z-stacks were created in Imaris x64 software (Version 7, Bitplane Scientific Solutions). Images were compiled using Adobe Photoshop CS4 without further digital manipulation.

2.2.3 Quantification Procedures and Statistical Analysis

2.2.3.1 Image Analysis

Quantification of nuclear AR protein expression, as well as membrane and cytoplasmic intensity of CXCR4 were undertaken using analysis scripts generated in Acapella® High Content Imaging and Analysis software (Version 2, PerkinElmer). Analysis was performed on cultures that had been fixed and processed for immunocytochemical labelling with anti-CXCR4 antibody or anti-AR antibody. Confocal images obtained on the Opera confocal microscope (20x objective) were used for analysis. Multiplexing of fluorophores for cellular labels for the nuclear region (Hoechst or DRAQ5™) or the cytoplasmic region (CellMask™ Blue) enabled the use of this software to identify regions of the cell for analysis. The Acapella® software analysis scripts are based on a series of “modules”; Nuclear detection (Hoechst or DRAQ5™ staining), was followed by cytoplasmic detection (CellMask™ Blue staining), and subsequent identification of regions of interest (ROI). The user specifies the specific channel the appropriate stain can be found in (e.g. CellMask™ Blue in channel 1, and DRAQ5™ nuclear staining in channel 2). Accordingly, the software modules for nuclear and cytoplasmic detection are designed to detect the borders of these regions based on variations in fluorescence intensity of the stained objects versus the background. The thresholds are adjusted according to the level of signal and background present in each image. The specified ROI was adjusted according to the aim of the analysis.

ROI (1) – Cytoplasmic intensity. The intensity of staining in the cytoplasmic region was quantified for the specified channel (e.g. Antibody staining of CXCR4, after stimulation with ligand). The raw intensity of the cytoplasmic region (defined as the cytoplasmic border of the cell minus the nuclear region) was analysed for each cell in the image, and output as the mean intensity per cell.

ROI (2) – Nuclear intensity. The intensity of staining was quantified in the nuclear region for the specified channel (e.g. Antibody staining of AR). The raw intensity
of the nuclear region was analysed for each cell in the image, and output as the mean intensity per cell.

ROI (3) – Cell membrane intensity. The intensity of staining at the cell membrane region was quantified for the specified channel (e.g. Antibody staining of CXCR4 in unstimulated cultures). For identification of the membrane region, an additional analysis module (“membrane region”) was incorporated into the body of the analysis script, subsequent to cytoplasmic detection. The membrane region module defines a “ring” mask at the outer region of the cell; starting on the outer border of the cell (which has been defined already in the cytoplasmic region), and extending that border into the interior of the cell. The distance to which this mask extends into the interior of the cell was a user-defined value.

This distance was selected based on the minimal region required to identify CXCR4 expressed on the cell membrane. Due to differences in cell morphology and spreading, the distance chosen for this ring region varied between cell lines. The chosen value was also varied between 2D and 3D cultures of the same cell line due to the more compact cell shape within spheroids generated in 3D culture.

This distance was kept consistent for each set of images analysed in parallel, in order to provide a consistent comparison between controls and treated wells. The raw intensity of the membrane region was analysed for each cell in the image, and output as the mean membrane intensity per cell across each image.

2.2.3.2 Image Analysis in 2D

For analysis of ROIs in 2D culture, the confocal plane used for analysis was that which provided a cross-section across the middle of most cells in the image. Any cells which lay above or beyond this cross-section were eliminated from analysis. Laser power and exposure settings were adjusted to achieve a clear picture in control wells, prior to use of identical settings in treatment wells. All treatments or time points to be used for comparison were imaged during the same session, using the same laser power and exposure settings.

2.2.3.3 Image Analysis in 3D

For analysis of ROIs in 3D culture, the confocal plane used for analysis was that which provided a cross-section of most spheroids in the image. Any spheroids that were out of focus or that lay above/below this cross-section were disqualified
from analysis. The laser power and exposure settings were adjusted to achieve a clear picture in control (untreated/vehicle controls), and identical settings were subsequently used on treatment wells. All treatments or time points used for comparison were imaged during the same session, on the same day, using the same microscope.

2.2.3.4 Quantification of Spheroid Size

Quantification of cell spheroid area from DIC images was performed using AxioVision Rel. 4.6.3 (Zeiss). Total spheroid area was obtained using the Area Tool, calculated based on spheroid diameter, and was compared across 4 different time points as a measure of spheroid growth.

2.2.3.5 Quantification of Stellate Projections

Analysis of stellate projections was undertaken using DIC images obtained from the IN Cell Analyser 2000 (GE Healthcare). Using the 10x objective, 4 fields of view were incorporated into one image using the IN Cell Developer software (Version 1.9, GE Healthcare). The resulting images were analysed for the number and length of stellate projections using AxioVision over 4 different time points (days 3, 5, 7 and 10). This was undertaken by manual application of the length measurement function, to trace the stellate processes from their insertion point at the edge of the spheroid to their maximal extension point. To ensure accurate measurements were achieved, only spheroids that were in focus within each image were included for analysis.

2.2.3.6 Statistical Analysis

Statistical analysis was carried out using Graph-Pad Prism (Version 5) and statistical significance for given variables was determined using either a paired t-test, in cases where analyses between different sample groups were to be undertaken. Normal distribution was assumed for simple comparisons (untreated versus treated), and parametric tests were employed for these analyses. Alternatively, repeated measures in the same group/sample were analysed for overall significance using one-way analysis of variance (ANOVA) via the Kruskal-Wallis test. The ANOVA results were then analysed using the Dunn’s Multiple Comparison test for post-hoc analysis, to determine the significance of differences between pairs within the data sets.
2.3 Cell Proliferation Assays

2.3.1 MTT Assay
To each well containing cells and culture medium, freshly prepared 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS; Cat. No. M2128) was added, to achieve a final concentration of 10% (v/v). Plates were incubated at 37°C, 5% CO₂ and 95% humidity for 1-2 hours, in which time the yellow MTT was metabolised by viable cells to form purple formazin crystals. Culture medium was then removed, prior to solubilisation of formazin crystals in 200 µl DMSO (Cat. No. 270431). Samples were transferred into clear 96-well plates for absorbance reading, in triplicate, at 570 nm, using the KC4 PowerWave™ (BioTek).

2.3.2 Alamar Blue™ Assay in 2D Culture
To assess cell proliferation, assays were performed in 384-well plates using Alamar Blue™ reagent (Cat. No. DAL1100, Life Technologies). Cells were plated in TC-treated Falcon 384-well plates (Cat. No. 6007558, PerkinElmer) at 100-350 cells/well in 50 µl complete medium per well and left to adhere for 16 hours at 37°C, 5% CO₂ and 95% humidity. Cells were then pre-treated with receptor inhibitors AMD3100 (5 µg/ml; Cat. No. A5602), anti-human CXCR7 antibody clone 9C4 (5 µg/ml; Cat. No. K0223-3, MBL International), anti-human CXCR4 antibody (10 µg/ml; Cat. No. MAB172, R&D systems), anti-human CXCR3 antibody (3 µg/ml; Cat. No. MAB160), bicalutamide (5µM; Cat. No. ICI176334M, Astra-Zeneca) or isotype controls IgG₁ (5 µg/ml; Cat. No. MAB002, R&D Systems) or IgG₂ (10 µg/ml; Cat. No. MAB004, R&D Systems). Cultures were incubated for 16 hours at 37°C, 5% CO₂ and 95% humidity prior to application of both ligands and inhibitors the following day (assay day 1). Ligands included SDF-1α (50 ng/ml; Cat. No. 350-NS-050, R&D Systems), ITAC (50 ng/ml; Cat. No. 672-IT-025, R&D Systems) or DHT (10 nM). Culture medium was refreshed every 2-3 days using a multichannel pipette. A baseline reading was taken 24 hours after plating (assay day 0), and readings were obtained on assay days 3, 7 and 10 through application of 5 µl of Alamar Blue™ per well, at a final concentration of 10% (v/v). After incubation for 4
hours at 37°C, 5% CO₂ and 95% humidity, plates were read on the EnVision® Plate Reader (PerkinElmer) using fluorescence excitation/emission settings of 530 nm/595 nm.

2.3.3 Alamar Blue™ Assay in 3D Culture

Assays were performed as described in 2.3.1 with the following modifications: each well of 384-well BD falcon plates was pre-coated with 15 µl of 70% (v/v) Matrigel (diluted in cold SFM), and left to set for 2 hours at 37°C, 5% CO₂ and 95% humidity prior to seeding cells on top at a density of 800 cells/well.

2.4 Ligand-Induced Receptor Signalling Assays

Cells were plated in 24-well TC-treated plates (Cat. No. CLS3527) at a density of 7,500-100,000 cells/well, and were left to adhere for 16 hours in complete medium. Cells were serum-starved for 16 hours in the absence or presence of CXCR4 inhibitor AMD3100 (5µg/ml) or anti-human CXCR7 function blocking antibody (5 µg/ml; Cat. No. K0223-3, MBL International). On the day of the assay, a 5x pre-dilution of SDF-1α (500 ng/ml) was prepared in SFM. The warmed SDF-1α solution was applied to each well to obtain a final concentration of 100 ng/ml, and incubated at 37°C, 5% CO₂ and 95% humidity. After 5-60 minutes of stimulation, samples were collected; culture medium was removed and 120 µl of ice-cold RIPA lysis buffer were added to each well. Samples were processed for SDS-PAGE and Western blot as described in Methods section 2.6.1-2.6.3.

2.5 Transwell® Cell Migration Assays

To investigate directional cell migration towards the CXCR4 ligand, SDF-1α, Transwell® cell migration assays (Cat. No. CLS3428) were employed. Two days prior to each migration assay, DU145, PC3 or LNCaP cells were seeded in 6-well plates at a density of 500,000 cells/well and left to adhere for 16 hours at 37°C, 5% CO₂ and 95% humidity. The following day, cultures were serum-starved for 16-24 hours in the presence or absence of AMD3100 (5 µg/ml).

Prior to seeding cells into Transwell® chamber assays, the underside of the Transwell® inserts were coated with 4 µg of fibronectin (Cat. No. 33016015, Life
Technologies) to encourage attachment of migrated cells. A 1 mg/ml fibronectin stock solution was diluted 1/12.5 in warmed PBS, and 5 µl of this solution was dispensed onto each insert and left to evaporate at RT. The inserts were then washed in PBS and equilibrated in SFM for 1 hour at 37°C, 5% CO₂ and 95% humidity before cells were seeded onto the prepared Transwell® inserts.

Cells were then harvested with Accutase® and seeded in 200 µl SFM, at a density of 80-150,000 cells per insert. Following addition of cells, 600 µl SFM was added to the lower chamber (with or without 400 ng/ml SDF-1α). Assay plates were incubated at 37°C, 5% CO₂ and 95% humidity for 6-16 hours.

Typically, cell migration was then quantified through staining with crystal violet. Migrated cells were fixed with 100% (v/v) methanol (Cat. No. LC 1115, RCI Labscan) for 10 minutes at -20°C, prior to application of crystal violet staining mixture containing 0.5% (w/v) crystal violet (Cat. No. C0775) in 20% (v/v) methanol. After 30 minutes, inserts were washed with deionised water to remove excess stain. The non-migrated cells on the upper surface of the insert were gently removed with a cotton swab, and the inserts were left to air dry. Cell migration was quantified by counting the number of migrated cells across 4 fields using the 20x objective on an Eclipse TS100 microscope (Nikon). Counts were averaged between 3 assay replicates. In other assays (Fig. 5.12), manual counting of migrated cells was not feasible due to the number of samples being quantified, instead, an MTT assay was used to quantify migrated cells. In this case, MTT solution was added to the lower well of the Transwell® chamber assay, as detailed in Methods section 2.3.1.

### 2.6 SDS-PAGE and Western Blot

#### 2.6.1 Cell Lysates

Cells were lysed in 100-200 µl ice-cold RIPA lysis buffer per well. The lysis buffer contained 70 mM TrisCl pH8 (Cat. No. T8404; titrated with HCl), 150 mM NaCl (Cat. No. S5886), 0.1% (w/v) SDS (Cat. No. 161-0301, Bio-Rad), 1% (v/v) Triton X-100 (Cat. No. X100), 0.5% (w/v) deoxycholic acid (Cat. No. D4297), 1mM EDTA (Cat. No. E9884). Protease inhibitors (Cat. No. 04693132001, Roche) were also added to lysis buffer to prevent protein degradation. Lysate
debris was cleared via centrifugation (20 minutes, 14,100 x g), and the supernatant was assayed for protein concentration (DC protein assay; Cat. No. 500-0112, Bio-Rad) prior to further analysis via Western blot.

For phosphorylated protein analysis, a phosphatase inhibitor cocktail (PhoSTOP; Cat. No. 04906845001, Roche) was also added to the lysis buffer as per manufacturer’s recommendations. Samples were transferred into microcentrifuge tubes, incubated on ice for 30 minutes and centrifuged at 14,000 x g for 30 minutes to pellet cell debris. The supernatant was transferred to a fresh tube and the sample was assayed for protein concentration.

2.6.2 Detergent Compatible (DC) Protein Assay

The DC protein assay (Cat. No. 500-0112, Bio-Rad) is based on the reaction of protein with alkaline copper tartrate solution and Folin reagent to produce colour change and measurable absorbance at 750 nm. Five microlitres of whole cell lysates (undiluted, diluted 1/2 and 1/5) were dispensed into 96-well plates (Cat. No. CLS3370), in triplicate. A standard curve of BSA protein (0.25-1.5 µg/µl) was prepared in the same plate. Twenty-five microlitres of the alkaline copper tartrate solution (reagents A & S) was added to protein samples, before addition of 200 µl Folin reagent (reagent B). Plates were then incubated at RT for 15 minutes before reading absorbance at 750 nm. The concentration of protein obtained from cell lysates was determined by comparing their absorbance reading to the BSA standard curve, to determine the corresponding protein concentration in µg/µl.

2.6.3 SDS-PAGE and Western Blot

Equal concentrations of samples were prepared in SDS-PAGE loading buffer containing 63 mM TrisCl pH 6.8, 13% (v/v) glycerol (Cat. No. G5516), 2.1% (w/v) SDS, bromophenol blue (Cat. No. B0126), 5% (v/v) β-mercaptoethanol (Cat. No. M6250), boiled for 10 minutes, and allowed to cool before loading onto SDS-PAGE gels (Table 2.4) alongside a molecular weight (MW) marker (Precision Plus All Blue protein standards; Cat. No. 161-0373, Bio-Rad). Electrophoresis was undertaken in SDS-PAGE running buffer containing 25 mM TrisCl pH8, 200 mM glycine (Cat. No. G8898) and 0.1% (w/v) SDS for approximately 1 hour at 170 V. After equilibration of blotting materials and the SDS-PAGE gels in Western blot transfer buffer (25 mM TrisCl pH8, 200 mM
glycine, 20% (v/v) methanol) for 30 minutes, the blotting apparatus (Criterion Blotter; Cat. No. 170-4071, Bio-Rad) was assembled and the Western blot was carried out at 30 V for 16 hours at 4°C. For Western blots of AR protein, 0.01% (w/v) SDS was added to the transfer buffer to facilitate transfer of higher MW proteins (Alimirah et al. 2006).

Table 2.4  Composition of SDS-PAGE gels.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>10% Resolving Gel</th>
<th>4% Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% (w/v) Acrylamide/Bis solution (Cat. No. 161-0145, Bio-Rad)</td>
<td>12.50 ml</td>
<td>3 ml</td>
</tr>
<tr>
<td>0.5 M Tris HCl pH 6.8</td>
<td>-</td>
<td>7.6 ml</td>
</tr>
<tr>
<td>1 M Tris HCl pH 8.8</td>
<td>18.75 ml</td>
<td>-</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.5 ml</td>
<td>300 μl</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
<td>25 μl</td>
<td>19.1 ml</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>250 μl</td>
<td>30 μl</td>
</tr>
<tr>
<td>Deionised H2O</td>
<td>15.5 ml</td>
<td>150 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 ml</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

Subsequently, membranes were incubated with blocking buffer; the blocking buffer typically consisted of 5% (w/v) non-fat milk powder (Diploma) in tris-buffered saline (TBS; 150 mM NaCl, 10 mM TrisCl pH 8). For AR studies, a less potent blocking buffer containing 0.1% (w/v) cold water fish skin gelatin (Cat. No. G7041) in TBS was employed, and for phosphorylated protein analysis 3% (w/v) BSA in TBS-Tween (TBST; TBS with 1% (v/v) Tween-20; Cat. No. P1379) was used.

After blocking, primary antibodies (Table 2.5) were diluted in blocking buffer and incubated with membranes for 16 hours at 4°C with agitation. After primary antibody incubation, membranes were washed 3 x 5 minutes in TBST and the appropriate secondary antibody was applied in blocking buffer for 1 hour at 4°C with agitation; either goat anti-mouse (Cat. No. 170-6516), or goat anti-rabbit
secondary antibody-horseradish peroxidase (HRP) conjugates (both 3 µg/ml; Bio-Rad). Subsequently, membranes were washed 3 x 5 minutes in TBST prior to application of chemiluminescence substrate (Cat. No. NEL103001EA, PerkinElmer) and imaging on Bio-Rad® Versa Doc™ imaging station.

**Table 2.5** Primary antibodies used for Western blot analysis.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Details</th>
<th>Species</th>
<th>Catalogue Number</th>
<th>Supplier</th>
<th>Concentration Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>Monoclonal Mouse anti-human</td>
<td>sc7305</td>
<td>Santa Cruz Biotechnologies</td>
<td>5 µg/ml</td>
<td></td>
</tr>
<tr>
<td>CXC4R1</td>
<td>Polyclonal Rabbit anti-human</td>
<td>Ab2074</td>
<td>Abcam</td>
<td>3 µg/ml</td>
<td></td>
</tr>
<tr>
<td>CXC7R1</td>
<td>Polyclonal Rabbit anti-human</td>
<td>ab38089</td>
<td>Abcam</td>
<td>5 µg/ml</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Monoclonal Mouse anti-human</td>
<td>A1978</td>
<td>Sigma-Aldrich</td>
<td>0.75 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Phospho-Akt(ser473)</td>
<td>Polyclonal Rabbit anti-human</td>
<td>9271</td>
<td>Cell Signaling Technology</td>
<td>5 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Total Akt</td>
<td>Polyclonal Rabbit anti-human</td>
<td>4685</td>
<td>Cell Signaling Technology</td>
<td>5 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Phospho-ERK 1/2</td>
<td>Polyclonal Rabbit anti-human</td>
<td>4377</td>
<td>Cell Signalling Technology</td>
<td>5 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Total ERK</td>
<td>Polyclonal Rabbit anti-human</td>
<td>4695</td>
<td>Cell Signalling Technology</td>
<td>5 µg/ml</td>
<td></td>
</tr>
</tbody>
</table>

To re-probe membranes for the loading controls (β-Actin or total Akt/ total ERK), membranes were incubated with mild stripping buffer (15% (w/v) glycine, 1% (w/v) SDS and 1% (v/v) Tween20, pH 2.2; Abcam), 2 x 7 minutes, washed in TBS (2 x 10min), and finally in TBST (2 x 5min) prior to re-blocking and probing for either β-Actin (0.75 µg/ml; Cat. No. A1978), total ERK (5 µg/ml; Cat. No.
4695, Cell Signaling Technology) or total Akt (5 μg/ml; Cat. No. 4685, Cell Signaling Technology).

Densitometric analysis of band intensity was performed using ImageLab software (Bio-Rad) and the density of the protein of interest was calculated in relation to the loading control (β-actin, total Akt or total ERK 1/2). This value was then normalised to the intensity value of the loading control for the untreated control, which was run alongside each set of samples within an experiment. Densitometric values were obtained by analysis of a one representative Western blot (n=1) from a complete set of n=3, an approach that was consistent between sets of experiments.

2.7 Cloning and Plasmid Propagation

2.7.1 Extraction of PSG5-AR

The PSG5-AR vector was kindly provided as a dried sample on filter paper by the Chang Laboratory (University of Rochester, New York; 2008). Elution of DNA from the filter paper was performed by placing the trimmed filter paper in 100 μl of sterile Tris-EDTA (TE) Buffer (1 mM EDTA, pH 8 and 10 mM TrisCl, pH 7.5) and vortexed for 2 minutes prior to storage for 16 hours at 4 ºC. The next day, the liquid was extracted from the filter paper by centrifugation. The isolated and purified DNA was then propagated in Escherichia coli (E.coli). DNA sequencing was used to confirm the identity of the plasmid and the presence of the AR gene.

2.7.2 Plasmid Preparation

Single colonies were inoculated into 5 ml Luria-Bertani (LB) broth, containing 1% (w/v) tryptone (Cat. No. T7293), 0.5% (w/v) yeast extract (Cat. No. Y1625), 1% (w/v) NaCl (Cat. No. S5886) in deionised H2O. Selection antibiotics were added depending on the selection marker present in the plasmid, either 50 μg/ml ampicillin (Cat. No. A1593,) or kanamycin (Cat. No. K1637). The cultures were growth for 16 hours at 37ºC with agitation.

The cultures were centrifuged at 5400 x g for 10 minutes at 4ºC to pellet the cells, and plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Cat. No. 27104, QIAGEN), according to the manufacturer’s instructions. Briefly, the cell pellet was resuspended in re-suspension buffer P1 and transferred to a
microcentrifuge tube, to which an equal volume of lysis buffer P2 was added and mixed. Buffer N2 was then added to neutralise the alkaline lysis buffer. The tube was then centrifuged for 10 minutes at 17,900 x g and the supernatant was applied to a QIAprep spin column and centrifuged for 1 minute at 17,900 x g to bind the DNA. The column was washed each with buffers PB and PE, before the DNA was eluted in 50 µl of nuclease-free H2O (Cat. No. 10977-015, Life Technologies). The concentration of DNA was then quantified using the NanoDrop spectrophotometer (Thermo Scientific). The typical yield per 5 ml culture was 15 µg DNA.

For larger scale preparation, the QIAfilter MidiPrep Kit (Cat. No. 12243, QIAGEN) was used to yield 100-300 µg DNA. Briefly, 150 ml of transformed for 16 hours cultures of E.coli were centrifuged (30 minutes, 3,000 rpm) and resuspended in 5 ml of P1 buffer, prior to addition of 5 ml of P2 buffer, mixing, and incubation for 5 minutes at RT. To initiate bacterial cell lysis, 5 ml of buffer P3 were added, and the preparation was mixed and incubated on ice for 15 minutes prior to centrifugation (5 minutes at 2,100 x g). The lysates were cleared using the QIAfilter Midi cartridge and applied to an equilibrated Qiagen-100 Tip in order to bind the DNA. The column was rinsed with wash buffer and the bound DNA was eluted with buffer QF. The eluant was precipitated with isopropanol, and resuspended in TE buffer containing 5 M NaCl prior to centrifugation to remove salt. The DNA was precipitated from the TE buffer using 100% (v/v) ethanol, incubated for 16 hours at -80ºC, centrifuged at 13,000 rpm for 30 minutes at 4ºC, and finally resuspended in 150 µl TE buffer. DNA concentration was quantified via NanoDrop, with a typical yield of 200 µg DNA per 150 ml bacterial culture.

2.7.3 Bacterial Transformation

The heat shock method of transforming DNA into bacteria was used to propagate plasmids. A 50µl aliquot of chemically competent α-Select DH5α E.coli (Cat. No. BIO-85046, Bioline) was defrosted on ice for 5 minutes before addition of 5 µl DNA mixture and gently mixed. The reaction was placed on ice for 30 minutes before a 30 second heat shock at 42ºC. The reaction was placed on ice for 2 minutes before addition of 200-500 µl of SOC medium, and was incubated at 37ºC for 1-3 hours for recovery. The transformed bacteria were then plated out on
a selective agar plate made with LB broth containing 1.5% (w/v) agar (Cat. No. A5306), and appropriate antibiotic selection: either 50 μg/ml ampicillin (Cat. No. A1593) or kanamycin (Cat. No. K1637). After 16 hours incubation at 37°C, the plates were visually assessed for colony growth, and single colonies of transformed clones were isolated by selecting a colony from the spread plate and streaking twice. Single colonies from the second streak plate were used to inoculate 5 ml LB broth containing selection antibiotics, and grown for 16 hours at 37°C with agitation.

2.7.4 Polymerase Chain Reaction (PCR)

PCR was used to prepare samples for DNA sequencing analysis, and to facilitate cloning of the AR into the pENTR™/D-TOPO® construct.

2.7.4.1 PCR for DNA Sequencing Analysis

2.7.4.1.1 Sequencing PCR for AR

DNA sequencing was used to confirm the identity of the PSG5-AR plasmid, and to confirm that products of a ligation reaction had successfully incorporated insert DNA into the vector construct. For this purpose, multiple primers were designed to span the length of the 2.7 kb open reading frame of the AR gene using Primer3 software (Rozen et al. 2000). Due to the length of the AR sequence, 7 separate AR forward primers were designed to provide complete coverage of the gene at approximately 300 base intervals throughout this sequence (Appendix A). A primer melting temperature of approximately 50°C was selected in order to be compatible with the sequencing polymerase Big Dye® Terminator (Life Technologies). The 5’ and 3’ ends of the AR gene were sequenced with M13 Forward/Reverse and T7 primers. Primers were synthesised by Geneworks and stock solutions were prepared in nuclease-free H2O.

The first step of DNA sequencing consisted of a sequencing PCR reaction. The DNA template was amplified using the AR sequencing primers in a PCR (Table 2.6). Big Dye® Terminator (v3.1, Cat. No. 4337454; Life Technologies) was utilised as a DNA polymerase in these reactions, to incorporate fluorescently-tagged nucleotides into the amplified DNA fragments for sequencing analysis. PCR cycling conditions for DNA sequencing reactions are listed in Table 2.7.
Table 2.6 Components of a DNA sequencing PCR.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Volume/amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>200 ng (vector/construct)</td>
</tr>
<tr>
<td>Forward primer</td>
<td>3.2 pmol</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>3.2 pmol</td>
</tr>
<tr>
<td>DNA polymerase: Big Dye® Terminator</td>
<td>1 µl</td>
</tr>
<tr>
<td>Big Dye® sequencing buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>Nuclease-free H₂O</td>
<td>-</td>
</tr>
<tr>
<td>Polymerase used</td>
<td>Big Dye® Terminator (Applied Biosystems)</td>
</tr>
</tbody>
</table>

Table 2.7 Cycling conditions used for sequencing PCR reactions.

<table>
<thead>
<tr>
<th>Cycling step</th>
<th>Temperature</th>
<th>Length of Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>9 °C</td>
<td>60 sec</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>96°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>3. Primer annealing</td>
<td>50°C</td>
<td>5 sec</td>
</tr>
<tr>
<td>4. Elongation</td>
<td>60°C</td>
<td>240 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Final extension</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Polymerase used</td>
<td>Big Dye® Terminator (Applied Biosystems)</td>
<td>-</td>
</tr>
</tbody>
</table>

Following the sequencing PCR, an Ethanol/EDTA/Sodium acetate precipitation step was performed to purify the amplified DNA from the remaining components of the PCR. The reaction product was added to a tube containing 2 µl of 3M sodium acetate (pH 5.2) and 2µl of 125mM EDTA (pH8). After briefly vortexing the solution, 50 µl of 100% (v/v) 200-proof ethanol (Cat. No. E7023) was added, mixed and incubated at RT for 15 minutes. The reaction was then centrifuged for 20 minutes at 22,000 x g at 4°C. The supernatant was removed and the 100% (v/v) ethanol wash step repeated. The pellet was then rinsed with 250 µl of 70% (v/v) ethanol, mixed, and centrifuged for 5 minutes at 22,000 x g at 4°C. The
supernatant was removed and the wash step was repeated, prior to air-drying the pellet. The sample was then submitted to the Griffith University DNA Sequencing Facility for analysis via capillary electrophoresis DNA sequencing. The capillary electrophoresis technique measured the fluorescent signal of the amplified products using a fluorescence peak trace, where the signal output corresponded to the nucleotide sequence of the sample. The sequences generated from this analysis were viewed and compiled using Applied Biosystems Sequence Scanner v1.0.

2.7.4.2 The Use of PCR to Clone AR for Ligation into Gateway® Vector

PCR was used to amplify the AR gene from the PSG5-AR transient expression vector, to be used for ligation into a vector suitable for stable gene expression. A tetracycline-inducible stable expression Gateway® vector was selected for this purpose, which required the cloning of the AR gene into an intermediate entry vector (pENTR™/D-TOPO®; Cat. No. K2435-20, Life Technologies) prior to transfer into the final stable expression vector (pTREX™-DEST30) (Cat. No. 12301016, Life Technologies).

2.7.4.2.1 Components of PCR Reaction for Gateway® Cloning

PCR conditions for amplification of AR from the PSG5-AR vector were optimised, and reaction components and cycling conditions are listed in Tables 2.8 and 2.9. The length of the AR insert (2.7 kb) required a DNA polymerase that was able to produce large amplicons, and DyNAzyme EXT polymerase (Cat. No. F-505S, Thermo Scientific) was selected for this purpose. The GC-rich nature of the AR resulted in the use of DMSO (5% v/v final concentration) during PCR to aid in denaturation of the template, to permit effective amplification of the gene. The dNTP mixture was sourced from Bioline (Cat. No. BIO-39044) and the sequences of primers used in these reactions are listed in Appendix A.
Table 2.8 Components of PCR reactions used for amplification of AR insert from PSG5-AR vector for use in Gateway® cloning.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Amount Per Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template (PSG5-AR)</td>
<td>0.75 ng</td>
</tr>
<tr>
<td>Forward primer (T7)</td>
<td>0.75 µM</td>
</tr>
<tr>
<td>Reverse primer (BGH)</td>
<td>0.75 µM</td>
</tr>
<tr>
<td>dNTP mixture</td>
<td>1 mM</td>
</tr>
<tr>
<td>DMSO</td>
<td>1 µl</td>
</tr>
<tr>
<td>DyNAzyme EXT reaction buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>DyNAzyme EXT DNA polymerase</td>
<td>0.5 U (0.1 µl)</td>
</tr>
<tr>
<td>Nuclease-free H₂O</td>
<td>to 20 µl</td>
</tr>
</tbody>
</table>

Table 2.9 PCR cycling conditions for AR amplification as part of Gateway® Cloning.

<table>
<thead>
<tr>
<th>Cycling Step</th>
<th>Temperature</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>98 °C</td>
<td>90 sec</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>98 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>60.8 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>4. Elongation</td>
<td>72 °C</td>
<td>80 sec</td>
</tr>
<tr>
<td>Cycles</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>
2.7.5 Restriction Enzyme Digestions

Restriction enzyme digests were used to cut DNA sequences during the DNA cloning process. These enzymes were used to linearise plasmids, excise portions of DNA or to create compatible ends for ligation of new sequences into the plasmid itself. Reaction conditions varied according to the restriction enzymes utilised (Table 2.10), and all restriction enzymes were sourced from New England Biolabs (NEB). Typically, after enzyme digestions, reaction products were analysed via 1.5% (w/v) agarose gel to separate DNA fragments and examine their size, and in some cases DNA fragments were purified by gel extraction for DNA ligation.

Table 2.10 Restriction enzyme digestions: components and conditions used.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Reaction (A)</th>
<th>Reaction (B)</th>
<th>Reaction (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asc1</td>
<td>Not1</td>
<td>Bgl II and BamHI</td>
</tr>
<tr>
<td>Asc1</td>
<td>0.3 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Not1</td>
<td>-</td>
<td>0.3 µl</td>
<td>-</td>
</tr>
<tr>
<td>Bgl II</td>
<td>-</td>
<td>-</td>
<td>2 µl</td>
</tr>
<tr>
<td>BamHI</td>
<td>-</td>
<td>-</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reaction buffer</td>
<td>2 µl NEB buffer 3</td>
<td>2 µl NEB buffer 4 (with BSA)</td>
<td>2 µl NEB buffer 3 (with BSA)</td>
</tr>
<tr>
<td>DNA</td>
<td>2 µg of pENTR™/D-TOPO® vector</td>
<td>500 ng of reaction product from reaction (A)</td>
<td>500 ng of either: (i) PSG5-AR, or (ii) pENTR™/D-TOPO®-linker</td>
</tr>
<tr>
<td>Nuclease-free H₂O</td>
<td>to 20 µl</td>
<td>to 20 µl</td>
<td>to 20 µl</td>
</tr>
<tr>
<td>Reaction conditions</td>
<td>16 hrs at 37°C</td>
<td>16 hrs at 37°C</td>
<td>2 hrs at 37°C</td>
</tr>
</tbody>
</table>

Reactions (A) and (B) were sequential digestions required to create compatible base-pair overhangs for ligation of the DS-linker into the pENTR™/D-TOPO® vector. Reaction (C) was used to digest the PSG5-AR (i) and pENTR™/D-TOPO®-linker (ii) vectors, to excise the AR sequence from PSG5-AR, and generate compatible base-pair overhangs for ligation into the pENTR™/D-TOPO®-linker vector. NEB = New England Biolabs.

2.7.6 Extraction of DNA from Agarose Gels

The DNA fragment products of restriction enzyme digestions were analysed via agarose gel electrophoresis, prior to gel extraction and purification of the
fragments. Restriction enzyme reaction products were analysed using 1.2% (w/v) agarose gel electrophoresis for 40 minutes at 90 mV, in Tris-Acetate-EDTA (TAE) Buffer containing 40 mM TrisCl pH 8 (Cat. No. T8404, titrated with HCl) 1 mM EDTA (Cat. No. E9884), 5.7% (v/v) glacial acetic acid (Cat. No. A9967). A 1 kb DNA ladder (Cat. No. 10787-018, Life Technologies) was included on each gel as a DNA fragment size reference. Ethidium bromide was used to stain the DNA and permit visualisation under UV light. The band of interest was excised from the gel and the DNA of interest was purified using QIAquick Gel Extraction Kit (Cat. No. 28704, QIAGEN). The gel slice was placed in a microcentrifuge tube and dissolved in buffer QG at 50 ºC for 10 minutes. One gel volume of isopropanol was added and mixed. The solution was applied to a QIAquick column and centrifuged for 1 minute at 22,000 x g. The column was washed once each with buffer QG and PE, and centrifuged for 1 minute at 22,000 x g. The DNA was eluted in 30-50µl of nuclease-free H2O and DNA concentration (µg/µl) was determined using a NanoDrop Spectrophotometer.

2.7.7 DNA Ligation

DNA ligase was employed to re-circularise plasmid sequences, to mediate the re-attachment of compatible DNA overhangs between insert and vector samples subsequent to their digestion with restriction enzyme/s.

2.7.7.1 Reaction Protocol and Components

DNA ligation reactions were performed using T4 DNA ligase (Cat. No. M0202S, NEB), with reaction components listed in Table 2.11. The ligation reaction was heated to 65ºC for 5 minutes and allowed to cool, prior to the addition of the ligase. A variety of molar ratios of insert: vector DNA were tested (ratios of 0.5:1–4:1), whilst the combined concentration of DNA in the reaction was maintained at 15-50 ng. Reactions were incubated at 16ºC for 16 hours, prior to ligase deactivation at 65ºC for 10 minutes. The ligation mixture was then transformed into E.coli for propagation and subsequent analysis.
Table 2.11 Components of DNA ligation reactions.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Ligation Reaction (A)</th>
<th>Ligation Reaction (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert DNA</td>
<td>10 ng of DS-linker</td>
<td>25 ng of AR insert (purified from PSG5-AR digestion with Bgl II and BamHI)</td>
</tr>
<tr>
<td>Vector DNA</td>
<td>10 ng of pENTR\textsuperscript{TM}/D-TOPO\textsuperscript{®} vector (digested with AscI and Not)</td>
<td>25 ng of pENTR\textsuperscript{TM}/D-TOPO\textsuperscript{®}-linker vector (digested with Bgl II and BamHI)</td>
</tr>
<tr>
<td>T4 DNA ligase buffer</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease-free H\textsubscript{2}O</td>
<td>to 20 µl</td>
<td>to 20 µl</td>
</tr>
<tr>
<td>Reaction conditions</td>
<td>16 hrs at 16°C</td>
<td>RT for 30 min</td>
</tr>
</tbody>
</table>

Reaction (A) was used for ligation of the DS-linker into the digested pENTR\textsuperscript{TM}/D-TOPO\textsuperscript{®} vector, resulting in the pENTR\textsuperscript{TM}/D-TOPO\textsuperscript{®}-linker construct. Reaction (B) was used for ligation of the digested and purified AR insert into the digested pENTR\textsuperscript{TM}/D-TOPO\textsuperscript{®}-linker vector, resulting in the pENTR\textsuperscript{TM}-AR construct.

2.7.7.2 Oligonucleotide Linkers: Addition of Restriction Enzyme Sites into Gateway\textsuperscript{®} Vector

Compatible restriction enzyme sites for NotI and AscI were incorporated into the pENTR\textsuperscript{TM}/D-TOPO\textsuperscript{®} vector to allow the AR gene to be directionally cloned into the construct. Oligonucleotide linkers were utilised for this purpose, consisting of short segments of double stranded DNA, designed to contain the restriction sites of interest.

2.7.7.2.1 Oligonucleotide Linker Design

Oligonucleotide linkers were designed to maintain a consistent codon reading frame within both the AR gene and the final pTREX\textsuperscript{TM}-DEST30 vector. These linkers were also designed to anneal together with compatible nucleotide overhangs which would be compatible for ligation into pENTR\textsuperscript{TM}/D-TOPO\textsuperscript{®}, after digestion of the vector with the NotI and AscI restriction enzymes. Linkers were synthesised by Geneworks, and sequences are shown in Table 2.12.
Table 2.12 Oligonucleotide linker sequences.

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Nucleotide Sequence 5’-3’</th>
<th>Melting Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Linker</td>
<td>GGCGCTAGATCTACTGGATCCAGG</td>
<td>62.6°C</td>
</tr>
<tr>
<td>Reverse Complementary</td>
<td>CGCGCCTGGATCCAGTAGATCTAGC</td>
<td>62.6°C</td>
</tr>
</tbody>
</table>

2.7.7.2.2 Generating the Double-Stranded (DS)-Linker

The forward and reverse linkers were reconstituted in 100 µM stocks in nuclease-free H$_2$O, and 3 µl of each of the forward and reverse linker stock solutions were added to annealing buffer (10 mM TrisCl pH8, 50 mM NaCl, 1 mM EDTA) in a total reaction volume of 20 µl. The annealing reaction was incubated at 95°C for 10 minutes, and cooled to RT prior to use in a ligation reaction with pENTR™/D-TOPO®. The structure of the annealed linker is shown in Fig. 2.1.

![Figure 2.1 Structure of the annealed DS-linker.](image)

The annealed base sequence is shown in bold with matching base pairs linked by vertical lines. The restriction enzyme sites are highlighted in boxes, filled boxes indicate sites required for insertion of the AR sequence and unfilled boxes indicate sites required for insertion of the linker into the pENTR™/D-TOPO® vector.

2.7.7.2.3 Ligating the DS-Linker into pENTR™/D-TOPO® Vector

The pENTR™/D-TOPO® vector was placed in sequential digestion reactions with the NotI and AscI restriction enzymes. The digestion product was gel purified and placed in a ligation reaction with DS-linker. The incorporation of the linker into the vector was confirmed by restriction digestion with Bgl II and BamHI, as the restriction sites for these enzymes was only present in pENTR™/D-TOPO® vectors that had successfully incorporated the linker sequence. This produced linearised plasmid fragments at approximately 2.7 kb when reaction products where analysed via agarose gel electrophoresis, as
opposed to the uncut, circularised plasmid evident as a blurred group of bands near the top of the gel (Fig. 5.5).

2.7.7.2.4 Cloning AR into the pENTR<sup>TM</sup>/D-TOPO®-Linker Vector

In separate reactions, the PSG5-AR vector and the pENTR<sup>TM</sup>/D-TOPO®-linker vector were placed in double digestions with both Bgl II and BamHI restriction enzymes. Products of the restriction digests were gel purified and placed in a ligation reaction. The ligation reaction products were then transformed into *E.coli* for propagation, plasmid purified and analysed by restriction digest and DNA sequencing to confirm incorporation of the full length AR gene, in the correct orientation.

2.7.7.2.5 Clonase<sup>TM</sup> Reaction: Producing pTREX<sup>TM</sup>-DEST30-AR

The pTREX<sup>TM</sup>-DEST30 tetracycline inducible expression vector allows utilisation of Gateway<sup>®</sup> technology, in which site-specific recombination reactions are used in the place of traditional DNA ligation reactions. Once the AR gene had been successfully cloned into the pENTR<sup>TM</sup>/D-TOPO®-linker vector, this construct was placed in a Clonase<sup>TM</sup> LR recombination reaction with the pTREX<sup>TM</sup>-DEST30 destination vector to mediate the transfer of AR between these two vectors. Each Clonase<sup>TM</sup> LR reaction consisted of 60 ng pENTR<sup>TM</sup>-AR, 150 ng pTREX<sup>TM</sup>-DEST30, 2 µl Gateway<sup>®</sup> LR Clonase<sup>TM</sup> II enzyme mix (Cat. No. 11791-100; Life Technologies) and 8 µl of TE buffer. The reaction mixture was mixed gently and incubated at RT for 1 hour, prior to deactivation with 2 µg of proteinase K (37°C for 10 minutes). The reaction product was then propagated in *E.coli*, purified and analysed via restriction enzyme digestion and DNA sequencing, to confirm the presence of the full-length AR gene in the correct orientation.
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2.8 Transfections of PCa Cells with AR Expression Vector

2.8.1 PSG5-AR Transient Cell Transfections

DU145 or PC3 cells were seeded in 6-well plates (2,000-3,000 cells/well) to reach 70-80% confluency the next day. Culture medium was removed and replaced with Opti-Minimum Essential Medium (MEM), a serum-free transfection medium preparation (Cat. No. 31985070, Life Technologies). For each well, 4 µg DNA (PSG5-AR or PSG5-Empty vector) was mixed with 8 µl Lipofectamine® 2000 (Cat. No. 11668019, Life Technologies) and incubated for 20 minutes at RT. The complex was then added directly to cells, and incubated for 7 hours at 37ºC, 5% CO₂ and 95% humidity. Cells were then harvested and re-seeded in complete medium, left to recover at 37ºC, 5% CO₂ and 95% humidity for approximately 9 hours. Subsequently, cells were serum-starved for 16 hours in the presence of DHT (10 nM) or the AR antagonist bicalutamide (5 µM) where indicated prior to use in assays. In later experiments as indicated below, the transfection reagent was changed to Lipofectamine® Low Toxicity (LTX; Cat. No. 15338-100, Life Technologies) to minimise the impact of the transfection reaction on cell viability.

2.8.2 Cell Transfections with Inducible Expression Vector

The efficiency of tetracycline-inducible expression was influenced by the relative expression levels of both the Tet-repressor (pcDNA™6/TR) and the gene of interest (AR; in the pTREX™-DEST30 vector). To test which ratios of the vectors provide the maximal window between un-induced and tetracycline-induced gene expression, the pTREX™-DEST30-LacZ vector was used to transfect cells in combination with the pcDNA™6/TR vector (Cat. No. V102520, Life Technologies). The pTREX™-DEST30-LacZ vector allows for expression of the easily detectable gene product, β-Galactosidase, which was measured using the chlorophenol red-beta-D-galactopyranoside (CRPG) assay (Felgner et al. 1994; Flores 2005). After optimisation, the optimised conditions can be used for transfection of cells with the pTREX™-DEST30-AR vector, in conjunction with pcDNA™6/TR.
2.8.2.1 Optimisation of Vector Co-Transfection Ratios

The pTREX™-DEST30-LacZ vector (supplied with Cat. No. 12301016, Life Technologies) was used in combination with the pcDNA™6/TR vector to determine the optimal ratios of these vectors for induction of gene expression in each cell line. Measurement of β-Galactosidase production in cells co-transfected with both pTREX™-DEST30-LacZ and pcDNA™6/TR permitted a simple measurement of un-induced and tetracycline-induced gene expression, to determine the vector ratios which provided optimal gene repression/induction.

DU145 or PC3 cells were seeded in 12-well TC-treated plates (Cat. No. CLS3512) at 120,000 cells/well and incubated for 16 hours at 37°C, 5% CO₂ and 95% humidity. Cells were then co-transfected with pcDNA™6/TR and pTREX™-DEST30-LacZ using Lipofectamine® LTX reagent. In a sterile tube, 2 µg of DNA (consisting of 3:1-12:1 ratios of pcDNA™6/TR: pTREX™-DEST30-LacZ) was mixed with 4 µl Lipofectamine® LTX in 500 µl OptiMEM per well and incubated at RT for 20 minutes. Cell culture medium was removed and fresh OptiMEM transfection medium was applied (1 ml/well), to which the DNA/Lipofectamine® mixture was added, followed by incubation at 37°C, 5% CO₂ and 95% humidity for 5 hours. Culture medium was then changed to complete medium for recovery for 16 hours. The following day, cells were either left untreated or incubated in the presence of 1 µg/ml Tetracycline for 24 hours. Culture medium was aspirated and 400 µl of lysis Buffer (25 mM TrisCl pH 8, 0.1% (v/v) Triton X-100) were applied. After incubation at RT for 15 minutes, samples were collected in 1.5 ml microcentrifuge tubes and incubated at -70°C for 15 minutes. Samples were then thawed at RT for 20 minutes, and tested in a CRPG assay in triplicate using 96-well untreated clear plates (Cat. No. CLS3370). To each well, 100 µl of 2x CRPG assay buffer were applied (18 mM MgCl₂, 204 mM β-mercaptoethanol in 160 mM Sodium Phosphate Buffer, pH 7.3). This was then combined with 60 µl of each lysate sample and 20 µl deionised water. Finally, 20 µl of CRPG (20 mM in deionised water; Cat. No 59767) were added and mixed. After incubation at 37°C, 5% CO₂ and 95% humidity for 45 minutes, absorbance was measured at 570 nm on the KC4 PowerWave™.
2.8.2.2 Transfections of Cells with Inducible pTREX\textsuperscript{TM}-DEST30-AR

In these experiments, Lipofectamine\textsuperscript{®} LTX was used to minimise the impact on cell viability that was originally observed after transfection with Lipofectamine\textsuperscript{®} 2000. DU145 or PC3 cells were seeded into T75 cell culture flasks at densities of 2.5-3 million per flask and left to adhere for 16 hours at 37°C, 5% CO\textsubscript{2} and 95% humidity. The next day, cells were approximately 80% confluent, and culture medium was replaced with 15 ml Opti-MEM. In a separate tube, 15 µg DNA (a 6 µg:1 µg ratio of pcDNA\textsuperscript{TM}6/TR and pTREX\textsuperscript{TM}-DEST30-AR vectors) was mixed with 15 µl Plus reagent and incubated for 5 minutes at RT. Subsequently, 45 µl of Lipofectamine\textsuperscript{®} LTX was added to the mixture and incubated for 30 minutes at RT. The complex was then added directly to cells and transfections were incubated for 16 hours at 37°C, 5% CO\textsubscript{2} and 95% humidity. Cells were then harvested and re-seeded in 12-well plates (150,000 cells/well), left to recover in complete medium at 37°C, 5% CO\textsubscript{2} and 95% humidity for approximately 9 hours. Subsequently, cells were serum-starved in the presence of inhibitors or controls as indicated (0.75 µg/ml Tetracycline; 5 µg/ml AMD3100; 10 nM DHT; 5 µM bicalutamide) and analysed via Transwell\textsuperscript{®} cell migration assay, immunocytochemistry or Western blot the next day.

2.8.2.3 Selection Antibiotic Sensitivity of Cells for Stable Selection

In order to select for stable pTREX\textsuperscript{TM}-DEST30-AR transfectants within DU145 and PC3 cell populations, the concentration of selection antibiotics required to kill non-transfected (non-resistant) cells was determined (Fig. 2.2). Non-transfected DU145 or PC3 cells were seeded into 12-well plates (8,000 cells/well for DU145; 10,000 cells/well for PC3), and incubated for 16 hours at 37°C, 5% CO\textsubscript{2} and 95% humidity. The following day, culture medium was replaced with complete medium containing G418 at concentrations between 100-1,200 µg/ml. Both culture medium and antibiotic selection were replenished every two days, and after ten days the percentage confluency was assessed by visual inspection of cultures using the Nikon Eclipse TS100 microscope.
Chapter Two: Materials and Methods

Figure 2.2 Resistance to selection antibiotics in non-transfected PC3 and DU145 cells. Cells were plated, left to adhere for 16 hours and treated with 100-1500 µg/ml G418 or 1-10 µg/ml Blasticidin for 10 days. Culture medium containing selection antibiotic was replenished every 2-3 days. On the final day, culture confluence was assessed by visual inspection on a Nikon Eclipse TS100 microscope. Concentrations for antibiotic selection of transfected cell populations were determined by values above the minimum concentration required to kill non-transfected cells. For PC3 cells, concentrations of 500 µg/ml (G418) and 7 µg/ml (Blasticidin) were chosen. For DU145 cells, concentrations of 800 µg/ml (G418) and 6 µg/ml (Blasticidin) were chosen.
2.9 Reverse Transcriptase-PCR (RT-PCR)

2.9.1 RT-PCR Primers

The expression of AR, CXCR4, PSA and GAPDH at mRNA level was studied using reverse transcriptase-PCR (RT-PCR). After extraction and purification of RNA from cell cultures, cDNA was synthesised and utilised as a template for PCR using gene-specific primers. Forward and reverse primers for AR, CXCR4, PSA and GAPDH mRNA sequences were generated using the Primer3 program (Rozen et al. 2000). The mRNA sequences for these genes were located using NCBI nucleotide, and primer sequences can be found in Appendix B.

2.9.2 RNA Extraction

DU145, PC3 or LNCaP cells were seeded in 6-well plates at densities of 300-400,000 cells per well and incubated at 37°C, 5% CO₂ and 95% humidity for 16 hours. The following day, cultures were serum-starved in the presence or absence of DHT (10 nM) and/or tetracycline (0.75 µg/ml) for 16 hours. Culture medium was removed and TRI reagent® was added directly onto cells at a volume of 1.5 ml per well. The preparation was passed through a 21-gauge needle for 5-10 times to adequately lyse cells. After incubation for 5 minutes at RT, preparations were centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube, and 0.1% (v/v) 1-bromo-3-chloropropane (BCP; Cat. No. B9673, Life Technologies) was added. The mixture was shaken vigorously and incubated for 10 minutes at RT before centrifugation at 12,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube, and nucleic acid was precipitated with isopropanol, and centrifuged at 12,000 x g for 8 minutes at 4°C. Subsequently, an ethanol precipitation was performed, and the RNA was dissolved in nuclease-free H₂O (~300 µl). To minimise the risk of contamination with RNases, disposable plasticware certified to be DNase and RNase free was used in the following experiments. The yield from each RNA extraction was assessed using the NanoDrop 1000 spectrophotometer (Thermo Scientific). The purity and quality of preparation were assessed by agarose gel electrophoresis and visualised using ethidium bromide, in order to observe whether intact 18S and 28S bands were present. Sharp, intact bands indicated a purified, non-degraded RNA preparation.
2.9.3 DNAse Digestion

An additional step was included to eliminate any residual DNA in the RNA sample, using the Turbo DNA-free™ kit (Cat. No. AM1907, Life Technologies). Briefly, 0.1 volumes of 10x TURBO DNase buffer and 1 µl TURBO DNase were added to 50 µl of RNA solution and incubated for 20-30 minutes at 37°C. The reaction was resuspended in DNase inactivation reagent (0.1 volumes). The solution was mixed well, incubated for 2 minutes at RT, while mixing every 30-45 seconds. After centrifugation at 10,000 x g for 1.5 minutes, the supernatant containing purified RNA was transferred to a fresh tube.

2.9.4 cDNA Synthesis

The purified RNA samples were then used to synthesise cDNA using a reverse transcriptase enzyme. A reverse transcriptase reaction was prepared using High Capacity RNA to cDNA kit (Cat. No. 4387406, Life Technologies). In each reaction, 2 µg of purified RNA was mixed with kit components including 10 µl reverse transcriptase buffer mix, 1 µl reverse transcriptase enzyme mix, and nuclease-free H2O to a total volume of 20 µl. Samples were then placed in a PCR thermocycler, and incubated at 37°C for 60 minutes followed by a 95°C incubation for 5 minutes to deactivate the enzyme.

2.9.5 RT-PCR Reaction

The expression of CXCR4, AR, PSA or GAPDH at an mRNA level was then measured by performing PCR reaction on the cDNA samples. Following completion of cDNA synthesis, the cDNA was used as a template for gene amplification in an RT-PCR reaction. The PCR reaction components were as listed in Methods section 2.7.4.1, with the following modifications; RT-PCR primers were used to measure expression of CXCR4, AR, PSA or GAPDH (see Appendix B) using Crimson Taq DNA polymerase (Cat. No. M0324S, NEB). Reaction products were analysed via 1% (w/v) agarose gel electrophoresis and visualised using ethidium bromide.
3 Chapter Three: The Effect of 3D Cell Culture on AR Regulation in PCA
Chapter Three: The Effect of 3D Cell Culture on AR Regulation in PCa

Results from this chapter are included in the published paper “In vivo biomarkers are preserved in 3D cultures of Prostate Cancer.” Windus, L.C. Kiss, D.L. Glover, T. Avery, V.M. Experimental Cell Research. July 2012. 318: 2507-2519.

3.1 Introduction

Androgens are the major steroid hormone regulators of male development and the maintenance of sexual characteristics. These hormones bind to the AR on prostate cells, and mediate actions such as the regulation of cell growth, apoptosis, protein secretion and differentiation (Falkenstein 2000; Kaarbo et al. 2007). The classical steroidal mode of action for AR involves initiating the transcription of AR-target genes, although it can also elicit effects that do not rely upon changes to transcriptional activity, known as non-genomic actions (Falkenstein 2000). Those include calcium mobilisation, activation of kinase cascades, the formation of FAK complexes, cytoskeletal re-arrangement and exocytosis of PSA (Hatzoglou et al. 2005; Kampa et al. 2002; Papakonstanti et al. 2003).

Evidence suggests that the progression to CRPC can be accompanied by a reactivation of the AR signalling pathway despite low levels of circulating androgens (Bluemn 2012; Debes et al. 2002; Javidan et al. 2005; Nieto et al. 2007). There are various mechanisms through which this has been proposed to occur, such as through AR gene amplification (Nieto 2007) or mutations (Suzuki 2003). Although the exact mechanisms have not yet been defined, it is now widely accepted that AR activity is maintained in CRPC despite low levels of androgens present in the circulation.

The expression of AR in commonly used in vitro models of androgen-insensitive, CRPC is still subject to debate. Initial investigations into AR expression in the DU145 and PC3 cell lines concluded they were AR-negative (Tilley 1995; Tilley 1990), whilst another group reported PC3 to express low levels of AR mRNA (Culig 1993). Whilst Buchanan and colleagues (2004) showed that PC3 sub-populations express endogenous AR mRNA and protein, Alimirah and colleagues (2006) demonstrated that a low level of AR protein could be detected in both DU145 and PC3 cell lines using optimised Western blotting methods. Interestingly, no PSA production was detected which suggests only partial functionality of the receptor in these cell lines (Alimirah et al. 2006). However,
other laboratories still maintain that DU145 and PC3 do not express AR (Harma et al. 2010). Androgen-insensitive derivatives of the LNCaP cell line that retain AR expression have been previously established through culturing in low androgen conditions (subline C4-2) (Wu 1994), however unfortunately these cell lines were not available upon request.

Here, the regulation of AR was investigated in both androgen-sensitive LNCaP and androgen-insensitive PC3 and DU145 PCa cell lines. Both 2D and 3D culture methods were used in these studies, to determine the effect of spheroid formation on the activity and distribution of AR. The interactions between cells and the ECM in 3D culture created an environment which was found to more effectively recapitulate that found in vivo (Nyga et al. 2011; Yamada 2007), and was capable of producing 3D structures and phenotypes that was distinctly different from traditional 2D monolayers (Windus et al. 2012). In the case of cancer cell lines, culturing with these matrices facilitated the formation of spheroid structures (Harma et al. 2010). Whilst 2D cultures of PC3 cells were found to be AR-negative, it is reported here that PC3 cells grown in 3D culture displayed an endogenous up-regulation of AR protein expression. Our results suggest that this expression of AR is:

1. Due to the structural changes in 3D culture, and cell-cell interactions of the cells rather than the growth factors present in Matrigel

2. Modulated by the Src/MAPK signalling pathway. However, the AR expressed in these cultures was found to display limited functionality, with no detectable PSA production or measurable impact of androgens on cell growth.

In contrast, the DU145 cell line maintained an AR-negative phenotype under both 2D and 3D culturing conditions. The LNCaP cell line was found to express androgen-responsive AR and PSA in both 2D and 3D conditions, and basal AR protein expression was not affected by inhibition of the Src/MAPK pathway. Collectively, these results suggest a differential regulation of AR protein expression and function in androgen-sensitive LNCaP and androgen-insensitive PC3 cells.
3.2 Results

3.2.1 AR Regulation in LNCaP and PC3 Cells

3.2.1.1 LNCaP Cells in 2D Culture

Firstly, 2D cultures of LNCaP cells were assessed for AR protein expression and function. As expected, LNCaP cells expressed AR protein, as shown by Western blot analysis (Fig. 3.1 A). The protein levels of AR were enhanced upon stimulation with DHT (5-10 nM; Fig. 3.1 A). This increase in AR protein has previously been shown to be a consequence of increased AR stabilisation in the presence of DHT (Waller et al. 2000). As AR protein expression was readily detectable in LNCaP cells, the localisation of the receptor was investigated using immunocytochemistry. AR protein expression as observed as diffuse staining in the cytoplasm and the nucleus (Fig. 3.1 B). After DHT treatment (10 nM, 24 hours), translocation of the AR to the nucleus was evident (Fig. 3.1 B’). The nuclear translocation of AR was quantified using script-based image analysis of AR intensity within the nucleus, and a statistically significant increase in the number of cells with nuclear AR localisation was demonstrated in the presence of 1-10 nM DHT (Fig. 3.1 C).
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Figure 3.1 LNCaP cells express androgen-responsive AR protein.
Cells were grown on tissue culture-treated plates in complete medium, prior to serum-starvation in the presence or absence of DHT (5-10 nM) for 30 hours. Samples were collected in lysis buffer and probed for AR protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. LNCaP cells were also analysed using an anti-AR antibody via immunocytochemistry, in control (B) and DHT-treated cultures (B’). AR is shown in green, phalloidin staining of F-actin in red and Hoechst staining of nuclei in blue. The distribution of AR in controls was observed as diffuse throughout the cytoplasm and nucleus, and a nuclear translocation of AR was observed in response to treatment with DHT. Scale bar = 60 µm. The translocation of AR to the nucleus in response to DHT was then quantified via script-based image analysis of AR intensity within the nucleus using Acapella® software (C). Nuclear translocation was expressed as a percentage of population with nuclei positive for AR staining. A significant increase in cells with nuclear AR localisation was observed in response to 1 nM, 5 nM or 10 nM DHT for 30 hours. p<0.01 Kruskal-Wallis test and **p<0.01 and *** p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=80-90. Results shown are representative of three separate experiments, with densitometry performed on representative data.

Next, the functionality of AR was investigated by analysis of cell growth and PSA production. The regulation of LNCaP cell growth by androgens was investigated by studying the effect of treatment with DHT and/or the non-steroidal AR antagonist bicalutamide over a 10 day period, using an Alamar Blue™ cell proliferation assay (Fig. 3.2 A). This resazurin-based reagent is reduced by living cells to yield a fluorescent product, and the signal obtained corresponds to the density of living cells in culture as a measure of metabolic activity (O’Brien 2000). LNCaP growth was enhanced in the presence of DHT (10 nM), a response which was inhibited by co-incubation with the anti-androgen bicalutamide (5 µM) (Fig. 3.2 A).
Although it was not found to be statistically significant, a trend towards reduced cell growth was observed in the presence of bicalutamide (Fig. 3.2 A). These results confirmed androgen-dependent growth regulation in the LNCaP cell line. As a transcription factor, AR binds to AREs in DNA to initiate transcription of target genes, such as PSA. Measurement of PSA in cell culture supernatants after 30 hours using a PSA ELISA demonstrated that PSA was expressed both constitutively and in response to DHT, indicating that AR was transcriptionally active in this cell line (Fig. 3.2 B). Although 10 nM DHT was sufficient to promote AR protein expression in LNCaP as observed via Western blot (Fig. 3.1 A), this concentration of DHT was not sufficient to stimulate additional PSA production at a level higher than the baseline PSA expression observed in this cell line (Fig. 3.2 B). This indicates that a higher dose of DHT, or a longer stimulation time, may be required to observe an enhanced PSA production in response to DHT.

![Figure 3.2](image)

**Figure 3.2** LNCaP cells display PSA production in 2D culture.

Regulation of cell growth was investigated using an Alamar Blue™ cell proliferation assay (A). Cells were incubated in culture medium containing 5% (v/v) CS-FBS, with DHT (10 nM) and/or the anti-androgen bicalutamide (5 µM) for 3-10 days. To quantify cell proliferation, Alamar Blue™ was incubated with cultures for 4 hours. Fluorescence readings were obtained on an EnVision® plate reader. A statistically significant (*p<0.05) increase in cell proliferation was observed at day 10 in the presence of 10 nM DHT. Cell culture supernatants were collected after 30 hours of incubation in the presence or absence of 10 nM DHT, and analysed via PSA ELISA (B). Statistical analysis performed using a paired t-test. Error bars denote S.E.M. Results shown are representative of three separate experiments.

### 3.2.1.2 3D Cultures of LNCaP Cells

Next, the effect of 3D culturing methods on the regulation of AR in LNCaP was investigated. In comparison to LNCaP cell cultures in 2D (Fig. 3.3 A), cells
grown in 3D using Matrigel for up to 11 days formed 3D spheroids with an irregular morphology (Fig. 3.3 B). Spheroid growth was quantified through measurement of spheroid area on days 3, 6, 9 and 11, showing a statistically significant increase in size over time (Fig. 3.3 C).

Figure 3.3 Growth of LNCaP cells in 3D culture generates spheroid structure.
Cells were seeded on tissue culture treated plates (2D) or wells containing Matrigel extracellular matrix (3D) and maintained in culture for up to 11 days. DIC images of 2D LNCaP culture is shown in (A). Cells cultured in 3D for 3, 6 and 9 days are shown in (B). Immunocytochemistry was also performed on LNCaP spheroids after 6 days in culture to demonstrate morphology; phalloidin staining of F-actin is shown in red and Hoechst staining of nuclei shown in blue (B’). Scale bars = 60 µm. Analysis of spheroid area was performed on DIC images, demonstrating a statistically significant increase in spheroid size over time (C). Error bars denote S.E.M. n=45-50. Results shown are representative of three separate experiments. p<0.01 Kruskal-Wallis test and *p<0.05, **p<0.01 *** p<0.001 post-hoc Dunn’s Multiple Comparison test.

The 3D cultures were found to express AR protein, which was enhanced after treatment with DHT (Fig. 3.4 A). As mentioned previously, this is likely the result of increased stabilisation of AR in the presence of DHT (Waller et al. 2000). Through immunocytochemical analysis of 3D cultures using an anti-AR antibody, AR was expressed as diffuse staining throughout the cytoplasm and nucleus in controls (Fig. 3.4 B). Upon treatment with DHT, a nuclear translocation response was observed (Fig. 3.4 B’). Script-based image analysis confirmed the nuclear translocation response, showing a statistically significant increase in nuclear AR
content in response to DHT (1-10 nM; Fig. 3.4 C). Testing of cell culture supernatants via ELISA showed 3D cultures to also produce PSA, and DHT treatment further enhanced production of this protein, demonstrating androgen-induced protein expression of this AR-target gene (Fig. 3.4 D). These results indicated that LNCaP cells cultured in 3D express functional AR protein.
Figure 3.4 3D cultures of LNCaP express functional AR.

LNCaP cells were cultured in 3D for 7 days, and serum-starved in the presence of 5-10 nM DHT for 30 hours. Whole cell lysates were analysed for AR protein expression via Western blot (A). The distribution of AR in controls (B) and in response to DHT (10 nM) treatment (B’) was assessed using an anti-AR antibody via immunocytochemistry. Density was calculated in relation to the β-actin loading control. AR labelling is shown in green, phalloidin staining of F-actin in red and Hoechst staining of nuclei in blue. Scale bar = 60 μm. Script-based image analysis confirmed a statistically significant increase in nuclear AR content in response to DHT in 3D culture (C). p<0.01 Kruskal-Wallis test and **p<0.01 *** p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=80-90. The production of PSA was measured in cell culture supernatants after 24 hours in culture, using a PSA ELISA (D). A statistically significant (P=0.0132) increase in PSA production was detected in response to DHT. *p<0.05 paired t-test. Error bars denote S.E.M. Results shown are representative of three separate experiments, with densitometry performed on representative data.
3.2.1.2.1 Regulation of AR by Signalling Pathways in LNCaP

AR can participate in cross-talk with PI3K/Akt, MAPK, Src and mTOR signalling pathways in PCa cells (Migliaccio et al. 2000; Mukherjee et al. 2008; Wang et al. 2007; Wu et al. 2010). The role of these pathways in the regulation of AR protein expression in LNCaP cells was investigated in 3D culture. To serve as a basis for comparison in androgen-insensitive PCa cells, the signalling pathway inhibitors wortmannin (PI3K/Akt inhibitor), rapamycin (mTOR inhibitor), PP2 (Src inhibitor), its inactive analogue PP3, or PD98059 (MAPK inhibitor) were applied to established LNCaP spheroids after 9 days in culture. Cells were serum-starved in culture medium containing inhibitors in the presence or absence of DHT (10 nM) for 30 hours, and the protein expression of AR was assessed (Fig. 3.5). DIC images of LNCaP spheroids in DMSO controls compared to inhibitors showed that rapamycin affected spheroid aggregation, and the health of cultures appeared to be adversely affected (Fig. 3.5 A). More rounded, symmetrical spheroids were generated after treatment with PD98059 (Fig. 3.5 A). Western blot analysis of AR protein in treated cultures showed no change in expression levels when any of these signalling pathways were inhibited (Fig. 3.5 B). These results suggest that AR protein expression in LNCaP cells can be maintained in the absence of activity through any of these pathways.

It was noted that inhibition of the mTOR (rapamycin) and PI3K/Akt (wortmannin) pathways enhanced AR protein expression, as detected via Western blot (Fig. 3.5 A). This increase in AR protein expression was also confirmed by immunocytochemistry, comparing control cultures (Fig. 3.5 C) with those incubated in the presence of rapamycin (Fig. 3.5 D-D’) and wortmannin (Fig. 3.5 E-E’).
Selective signalling pathway activity was not required to maintain AR protein expression in 3D cultures of LNCaP cells. LNCaP cells were cultured in 3D for 9 days and pre-treated with signalling pathway inhibitors including Src kinase inhibitor PP2 (5 μM), its inactive analogue PP3 (5 μM), mTOR inhibitor rapamycin (1 μM), PI3K/Akt inhibitor wortmannin (20 μM) or MAPK inhibitor PD98059 (10 μM) or DMSO vehicle (0.4%) for 24 hours. The next day, fresh inhibitors were added to cultures in the absence or presence of DHT (10 nM) for a further 30 hours. DIC images were obtained of spheroids after treatment with inhibitors (A), prior to collection of cell lysates for Western blot analysis of AR protein expression (B). Density was calculated in relation to the β-actin loading control. Using an anti-AR antibody, LNCaP cells were also processed for AR protein expression via immunocytochemistry after pre-treatment with DMSO vehicle (C-C’), rapamycin (D-D’) or wortmannin (E-E’). AR is shown in green, phalloidin staining of F-actin in red, and Hoechst staining of nuclei in blue. Scale bar = 60 μm. Results shown are representative of three separate experiments, with densitometry performed on representative data.
3.2.1.3 2D Cultures of PC3 Cells

A survey of the literature shows conflicting reports regarding the presence of AR in the androgen-insensitive DU145 and PC3 cell lines. To determine whether AR could be detected in these cell lines at a protein level, they were subjected to Western blotting and immunocytochemistry. To optimise the potential of detecting low levels of AR in these cell lines, two approaches were employed. Firstly, a modified Western transfer buffer was utilised to accommodate the transfer of higher-molecular weight proteins (AR is 110 kDa). Specifically, a lower methanol concentration was utilised (10% v/v, compared to standard 20%) and SDS was added (0.01% w/v) to the Western transfer buffer (Alimirah et al. 2006). Secondly, an antibody targeted to the conserved region of the AR sequence (amino acids 299-315) was used for Western blot detection of the AR protein. It is known that subtle variations within the AR sequence occur both in vivo and in vitro, particularly within the highly variable N or C terminal regions of the protein; and that antibodies targeted to the N-terminal, conserved and C-terminal regions of the AR protein show lower capacity to detect AR expression between PCa cell lines (Alimirah et al. 2006). PC3 cells cultured in 2D (Fig. 3.6 A) were lysed and probed for AR protein expression via Western blot (Fig. 3.6 B), or fixed and probed with an anti-AR antibody using immunocytochemistry (Fig. 3.6 C). In both instances, no AR protein was detected. However, in contrast to the findings of Alimirah and colleagues (2006), no detectable AR protein expression was found in 2D cultures of PC3 cells, either endogenously or in response to DHT treatment (Fig. 3.6).
Figure 3.6 PC3 cells do not express AR in 2D culture.
A DIC image of PC3 cells grown as a 2D monolayer is shown in (A). Scale bar = 100 µm. Cells were plated, serum-starved for 30 hours in the presence or absence of DHT (5-10 nM), and analysed for AR protein expression via Western blot (B). Density was calculated in relation to the β-actin loading control. 2D cultures were also probed for AR protein expression via immunocytochemistry using an anti-AR antibody (C). No AR protein expression was detected in 2D cultures of PC3 cells. AR labelling shown in green, phalloidin staining of F-actin shown in red, and Hoechst staining of nuclei is shown in blue. Scale bar = 60 µm. Results shown are representative of three separate experiments, with densitometry performed on representative data.

3.2.1.4 3D Cultures of PC3 Cells

PC3 cells cultured in 3D formed irregular-shaped spheroids after 7 days in culture, developing cellular projections that extended from the spheroid mass into the surrounding Matrigel (arrow, Fig. 3.7 A). Spheroid morphology in day 7 cultures was also observed via immunocytochemistry, using phalloidin to stain F-actin and Hoechst nuclear stain, in both a single confocal slice and as a 3D reconstruction (Fig. 3.7 B-B’). Analysis of spheroid area on days 3, 6, 9 and 11 demonstrated an increase in spheroid size over time (Fig. 3.7 C).
Figure 3.7 The development of spheroids in 3D cultures of PC3 cells.
PC3 cells were cultured in Matrigel and maintained for up to 11 days. DIC images of spheroids after 3 or 9 days of growth are shown in (A). Arrow indicates stellate projections. Scale bar = 100 µm. PC3 spheroids were fixed and analysed using immunocytochemistry after 7 days in culture (B). Phalloidin staining of F-actin is shown in green, and Hoechst staining of nuclei shown in red. A single confocal slice across the PC3 spheroid is shown in (B) and a 3D z-stack reconstruction is shown in (B’). Scale bar = 60 µm. Script-based image analysis of spheroid area demonstrated a statistically significant increase in spheroid size over time (C). p<0.01 Kruskal-Wallis test and *p<0.05, **p<0.01 *** p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=45-50. Results shown are representative of three separate experiments.
Interestingly, when PC3 cells were cultured in 3D, a clear induction of AR protein was observed (Fig. 3.8). Whilst these cells are AR-negative in 2D culture, Western blot analysis showed AR protein was present in 3D cultures and protein expression was enhanced upon treatment with DHT (5-10 nM, 24 hours; Fig. 3.8 A). This may be due to an increase in AR stabilisation in the presence of DHT, as reported previously in PCa cells (Waller et al. 2000). Analysis via immunocytochemistry confirmed the presence of AR in 3D cultures, and demonstrated a nuclear translocation of AR protein in response to DHT treatment (10 nM, 24 hours; Fig. 3.8 B).

![Figure 3.8](image)

**Figure 3.8** Expression of AR protein was induced when PC3 cells were cultured in 3D.

PC3 cells were cultured in the presence of Matrigel for 9 days. On the final day, cultures were serum-starved for 30 hours, in the presence or absence of DHT (10 nM). Whole cell lysates were collected and analysed via Western blotting for AR protein expression (A). Alternatively, cells in 96-well plates were fixed and AR protein expression was analysed via immunocytochemistry using an anti-AR antibody (B-B'). AR is shown in green, phalloidin staining of F-Actin shown in red, and Hoechst staining of nuclei shown in blue. Scale bar = 60 μm. Script-based image analysis of AR nuclear translocation was performed using Acapella® (C). The number of cells positive for AR nuclear staining was expressed as a percentage of the total number of cells per image. A significant increase in the number of nuclei with AR staining was demonstrated after treatment with DHT (1-10 nM) for 30 hours. Error bars denote S.E.M. n=80-90 spheroids. p<0.001 Kruskal-Wallis test and *** p<0.001 post-hoc Dunn’s Multiple Comparison test. Results shown are representative of three separate experiments, with densitometry performed on representative data.
Considering the induction of AR protein expression in 3D cultures of PC3 cells, the functionality of the AR in these cells was further investigated. Androgens can enhance the proliferation of androgen-sensitive LNCaP cells (Fig. 3.2), and although PC3 cells are commonly known to be androgen-insensitive, this re-expression of AR protein prompted an investigation into whether androgenic regulation of cell growth occurs in these cultures. PC3 cells cultured in 3D were treated with DHT and/or bicalutamide, and cell proliferation was assessed using Alamar Blue™ (Fig. 3.9). The rate of cell proliferation was not affected by incubation with DHT (10 nM) and/or bicalutamide (5 µM; Fig. 3.9), demonstrating that PC3 cells grown in 3D retain androgen-insensitive growth.

![Figure 3.9](image)

**Figure 3.9** PC3 cell growth was not affected by treatment with androgens in 3D culture.

PC3 cells were cultured for up to 10 days in culture medium containing 5% (v/v) CS-FBS, in the presence of DHT (10 nM) and/or bicalutamide (5 µM) where indicated. To assess cell proliferation, Alamar Blue™ was added to each well, and incubated for 4 hours prior to obtaining fluorescence readings on the EnVision® plate reader. Error bars denote S.E.M. Results shown are representative of three separate experiments.

To assess whether AR stimulation in PC3 cells was followed by the production of PSA, cell culture supernatants were assayed for PSA concentration using a PSA ELISA (R&D Systems). However, no PSA production was detected in cell culture supernatants after 24 or 48 hours in culture (n=6; data not shown). This suggests that, while AR protein expression was induced in 3D culture and was ligand-responsive, activation of this receptor by androgens was not followed by the production of PSA.
3.2.1.5 Regulation of AR Protein Expression in PC3 cells

3.2.1.5.1 Regulation of AR Protein in 3D Spheroids

Factors which may contribute to the endogenous enhancement of AR protein expression in PC3 cells were investigated by extracting the cells from their 3D culturing environment. Matrigel contains a variety of growth factors including EGF, insulin-like growth factor-1 (IFG-1) and transforming growth factor-beta (TGF-β), which can stimulate and up-regulate components of the AR signalling pathway (Bonaccorsi 2004; 2006; Hughes 2010; Zhu et al. 2008). It was therefore possible that the presence of these growth factors in Matrigel may contribute to the emergence of AR protein expression in PC3 cells. Another factor that may play a role in this re-expression of AR was the formation of spheroid structures in 3D culture, as morphological changes are known to correspond with profound changes to the expression of genes and proteins associated with cell growth and metabolism (Harma et al. 2010). Therefore, experiments were undertaken to distinguish whether the presence of Matrigel itself, in isolation of the spheroid structure, was sufficient to maintain the AR-positive PC3 phenotype observed in 3D culture.

PC3 cells were dissociated from established spheroids after 9 days of growth in Matrigel, and were re-seeded into two different conditions: 1. Directly onto TC-treated plates (2D), or 2. Into wells coated with a thin layer of Matrigel, an amount which was insufficient to support 3D spheroid formation, resulting in the growth of these cells in a 2D monolayer. AR protein expression was then analysed in these samples via Western blot. Whilst cells directly extracted from 3D conditions display AR protein expression (Fig. 3.10 A), the re-plating of cells after extraction in confluent 2D monolayers resulted in a loss of AR protein expression after 42 hours (Fig. 3.10 B). These populations displayed a reversion to the AR-negative status observed in 2D culturing conditions (Fig. 3.6), demonstrating that the induction of AR protein expression in 3D culture can be reversed by removal of these cells from Matrigel. When cells were re-plated in 2D conditions in the presence of minimal Matrigel, AR protein expression was significantly reduced (Fig. 3.10 C) when compared to 3D culture (Fig. 3.10 A). Therefore it was likely that the factors present in Matrigel alone are not sufficient to maintain this AR positive status observed in 3D culture. As dissociation of the
spheroids and the associated cell-cell interactions impacted profoundly on AR protein expression in PC3 cells, it was likely that the formation of the 3D spheroid structure itself played a major role in maintaining the AR-positive status.

Figure 3.10  AR protein expression was lost when PC3 spheroids were removed from 3D culture.
PC3 cells were maintained in 3D culture for 9 days. Cells were serum-starved in culture medium containing 5-10 nM DHT for 30 hours, lysed and probed for AR protein expression via Western blot (A). Alternatively, after 9 days in 3D culture, PC3 cells were extracted from 3D culture, and re-plated into a 2D monolayer; either directly onto TC-treated plates (B), or in the presence of a thin layer of Matrigel (C). Cells were left to adhere for 16 hours, serum-starved in culture medium containing 5-10 nM DHT for 30 hours, prior to lysis and analysis of AR protein expression via Western blot. The protein expression of AR was lost after re-plating the dissociated cells in 2D conditions. Density was calculated in relation to the β-actin loading control. Results shown are representative of three separate experiments, with densitometry performed on representative data.

3.2.1.5.2  Regulation of AR by Intracellular Signalling Pathways in PC3
The expression of AR can be regulated by various intracellular signalling pathways, including MAPK and PI3K/Akt (Lin 2001; Manin et al. 2002). The role of the MAPK, PI3K/Akt, Src and mTOR pathways in regulating AR protein expression in 3D cultures of PC3 cells was analysed using pathway inhibitors, Western blot and immunocytochemistry. After 9 days in 3D culture, established PC3 spheroids were treated with the following signalling pathway inhibitors: Src family kinase inhibitor PP2, its inactive analogue PP3, the mTOR inhibitor rapamycin, MAPK inhibitor PD98059 or PI3K /AKT inhibitor wortmannin for 24 hrs, prior to the addition of DHT (10 nM) for 30 hours in SFM. DIC images of spheroid morphology were obtained, prior to collection of cell lysates for analysis.
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of AR protein expression via Western blot. Cultures treated with PP2 displayed more organised cell clusters with reduced formation of stellate projections (Fig. 3.11 A). Western blot analysis showed AR protein expression was reduced in the presence of PP2, preventing the enhanced AR protein levels in response to DHT (Fig. 3.11 B). This was confirmed via immunocytochemical analysis for AR protein expression using an anti-AR antibody, which also revealed a lack of nuclear translocation in response to DHT treatment after incubation with PP2 (Fig. 3.11 D-D’). The inactive analogue PP3 did not affect spheroid morphology (data not shown), and had no impact on AR protein expression as seen by Western blot (Fig. 3.11 B). Rapamycin-mediated inhibition of the mTOR pathway reduced spheroid size, appearing to impact on cell viability (Fig. 3.11 A). In these cultures, AR protein expression was enhanced, as observed via Western blot (Fig. 3.11 B), and was confirmed with immunocytochemistry for AR (Fig. 3.11 E-E’). Altered spheroid morphology was also observed via immunocytochemistry of cells treated with rapamycin, featuring enlarged nuclei and a shrunken cytoplasm (Fig. 3.11 E-E’). These observations are consistent with cells undergoing apoptosis (Jia et al. 2012). Treatment with the MAPK inhibitor PD98059 resulted in a more elongated and spindle-like appearance of PC3 cells (Fig. 3.11 A). Western blot analysis of whole cell lysates after treatment with PD98059 showed that both AR protein expression and DHT-induced up-regulation of AR protein were inhibited (Fig. 3.11 B). Immunocytochemical analysis of cells treated with PD98059 using an anti-AR antibody confirmed a marked reduction in AR protein expression (Fig. 3.11 F-F’). In contrast, treatment with wortmannin did not affect AR protein expression, and the DHT-induced up-regulation of AR protein was maintained (Fig. 3.11 B). When considered together, these results suggest that endogenous AR protein levels were regulated by the Src/MAPK pathways in 3D cultures of PC3 cells.
Figure 3.11 AR protein expression was modulated by Src/MAPK inhibition in 3D cultures of PC3 cells.

PC3 cells were cultured in 3D for 9 days prior to addition of the inhibitors into culture medium for 16 hours; these include PP2 (Src kinase inhibitor, 5 µM), PP3 (inactive analogue of PP2, 5 µM), rapamycin (mTOR inhibitor, 1 µM), wortmannin (PI3K/Akt inhibitor, 20 µM), PD98059 (MAPK inhibitor, 10 µM) or DMSO (0.4%) vehicle control. The next day, culture medium was refreshed with SFM containing inhibitors, in the absence (-DHT) or presence (+DHT) of 10 nM DHT for 30 hours. DIC images of cultures were obtained prior to sample collection (A). Cell lysates were collected for Western blot analysis of AR protein expression (B). Density was calculated in relation to the β-actin loading control. Immunocytochemistry was performed using an anti-AR antibody, on cultures treated with DMSO vehicle (C-C'), PP2 (D-D'), rapamycin (E-E') or PD98059 (F-F'). Inhibition of the Src (PP2) and MAPK (PD98059) pathways reduced the protein expression of AR in 3D culture. AR is shown in green, phalloidin staining of F-actin in red, and Hoechst staining of nuclei in blue. Scale bar = 60 µm. Results shown are representative of three separate experiments, with densitometry performed on representative data.
3.2.2 Analysis of AR Protein Expression in DU145 Cells

In comparison to 2D monolayers (Fig. 3.12 A), the growth of DU145 cells in 3D culture facilitated the formation of symmetrical, rounded spheroids (Fig. 3.12 B). Analysis of spheroid area in DIC images demonstrated that spheroid growth increased over time (Fig. 3.12 B). The rounded spheroid phenotype of DU145 cells in 3D culture was also demonstrated via immunocytochemistry using phalloidin to stain F-actin, as illustrated in a single confocal slice (Fig. 3.12 D), and a z-stack reconstruction of the spheroid (Fig. 3.12 D’). Considering the effect of 3D culture on the protein expression of AR in PC3 cells, AR protein levels were investigated in DU145 cells. In 2D culture, no endogenous AR protein expression was detected, either at basal levels or in response to DHT (10 nM), as observed via Western blot (Fig. 3.12 E). DU145 cells in 3D culture were also found to be AR-negative (Fig. 3.12 F), demonstrating that the AR-negative status of DU145 cells remained consistent between these culturing environments.
Figure 3.12 DU145 cells were AR-negative in 2D culture and 3D culture.

A DIC image of DU145 cells cultured in 2D is shown in (A). The growth of spheroids on days 3, 6 and 9 in 3D culture is shown in (B). Scale bar = 100 μm. Script-based image analysis of spheroid area confirmed a statistically significant increase in spheroid size over time in culture (C). Error bars denote S.E.M. n=45-50 spheroids. p<0.01 Kruskal-Wallis test and *p<0.05, **p<0.01 ***p<0.001 post-hoc Dunn’s Multiple Comparison test. The rounded morphology of DU145 spheroids was further demonstrated using immunocytochemistry (D-D’). A confocal slice through a DU145 spheroid is shown in (D), and a z-stack reconstruction is shown in (D’). F-actin staining with phalloidin is shown in red (D) or green (D’), and Hoechst staining of nuclei is shown in blue (D) or red (D’). Scale bar = 60 μm. The protein expression of AR was analysed in whole cell lysates, after serum-starvation in the absence or presence of DHT (5-10 nM) for 30 hours prior to collection. Whole cell lysates collected from 2D (E) or 3D (F) culture were probed for AR protein expression via Western blot. No AR protein was detected in DU145 cells, in 2D or 3D culture. Density was calculated in relation to the β-actin loading control. Results shown are representative of three separate experiments, with densitometry performed on representative data.
3.3 Discussion

Here, the results of these studies show that spheroid morphology was divergent between PCa cell lines LNCaP, DU145 and PC3 grown in 3D culture conditions when compared to 2D monolayer cultures. Moreover, this is the first report to show re-expression of AR protein in PC3 cells grown in 3D, a profile that was not observed under 2D culturing conditions. Whilst the AR protein expressed in PC3 cells does not induce expression of PSA, AR protein levels in this cell line can be modulated by the Src/MAPK pathways. By comparison, inhibition of these pathways did not impact on AR protein expression in LNCaP cells. These findings suggest there was differential AR protein regulation and functionality observed between the androgen-insensitive PC3 and the androgen-insensitive LNCaP cell lines. Results from this chapter have been published in Experimental Cell Research (Windus et al. 2012).

3.3.1 PCa Cells in 2D and 3D Culture

We have shown here that culturing cells in 3D Matrigel cultures strongly supported the growth and differentiation of PCa cells. In comparison to their 2D counterparts, each cell line formed very different phenotypes. Utilising both DIC and fluorescence microscopy, we and others (Harma et al. 2010) have confirmed that, when grown in Matrigel, the PCa cell lines PC3, LNCaP and DU145 formed atypical spheroids with disorganised architecture. Consistent with a highly invasive cell phenotype, PC3 cells developed chain-like projections that were stellate in appearance, after 3 days in culture. These structures have been previously described by Harma and colleagues (2010). In addition, the formation of organised, round spheroids by DU145 cells in 3D culture was concurrent with observations reported by Harma and colleagues (2010).

3.3.2 Endogenous Up-Regulation of AR Protein in 3D Cell Cultures

This is the first report of 3D culturing methods inducing endogenous AR protein expression in PC3 cells, in populations that are AR-negative in a 2D culturing environment. To-date the conflicting literature concerning the expression of AR mRNA and protein in PCa cell-lines have been carried out primarily in 2D culture systems. Consistent with previous studies (Harma et al. 2010; Kaighn et al. 1979; Tilley 1995; Tilley 1990), DU145 and PC3 cells were not found to express
detectable levels of endogenous AR in 2D culture. Earlier studies have shown DU145 cells to be completely AR-negative and PC3 cells to express very low levels of normal AR (Culig 1993). In contrast, both PC3 and DU145 cells were reported to express low levels of endogenous AR mRNA and protein (Alimirah et al. 2006). However, despite replicating the conditions used in the study reported by Alimirah (2006), no detectable protein expression of AR in 2D cultures of DU145 and PC3 cells was observed in the current study.

3.3.3 AR Protein Expression in PC3 - Only in 3D Culture

The loss of AR protein in PC3 cells upon removal from 3D culture indicates that the 3D culturing environment was required for AR protein expression to be maintained. A thin layer of Matrigel in 2D monolayer culture was insufficient to maintain AR protein expression after cells were removed from 3D culture. It was likely that the 3D spheroid structure formation, cell-cell contact, and cell-ECM contact influenced the regulation of AR protein expression in PC3 cells.

The up-regulation of AR protein in 3D cultures of PC3 cells demonstrated that the ECM influences the behaviour and phenotype of PCa cells in vitro. The loss of AR protein expression in androgen refractory cells in 2D culture may occur, in part, due to the removal of these cells from their in vivo environment into a more artificial 2D culturing environment in vitro. This phenomenon has been reported with primary cultures of normal prostate epithelial cells, once extracted from the in vivo source, they de-differentiate and become androgen refractory (Peehl et al. 1989).

Major epigenetic changes are associated with the acinar differentiation seen in 3D culture (Harma et al. 2010). Three-dimensional culturing is known to result in profound changes to gene expression over a 5-13 day period when compared to cells cultured in 2D (Harma et al. 2010). Various genes involved in lipid/steroid metabolism, adhesion, ECM turnover and development/differentiation are up-regulated in 3D culture (Harma et al. 2010). Indeed, findings in our laboratory have shown that PC3 cells in 3D display a down-regulation of a6 integrin, and a complete loss of E-cadherin (Windus et al. 2012). Therefore, re-expression of AR protein in the PC3 cell line may be a direct consequence of the spheroid development process, and may only be maintained while this structure is intact.
3.3.4 Altered AR Functionality in PC3 Cells

Despite their expression of AR, the growth of PC3 cells in 3D culture remained androgen-insensitive. The addition of DHT, or the anti-androgen bicalutamide, did not affect cell growth. This was in contrast to the response of LNCaP cells, in which cell growth was enhanced upon addition of DHT in an AR-dependent manner. Evidently, the up-regulation of AR protein in 3D culture was not sufficient to provide an androgen-sensitive phenotype in PC3 cells.

The lack of PSA production in 3D cultures of PC3 cells indicates that the downstream effects of AR stimulation in androgen-sensitive PCa cells do not necessarily correspond to those observed in androgen-insensitive cells. The AR protein in PC3 cells was observed to undergo nuclear translocation in response to DHT, without functionally inducing PSA production. Further investigations into AR transcriptional activity are required in PC3 cells, to determine whether the expression of other AR-regulated genes can be detected in 3D culture. Previously, Alimirah and colleagues (2006) have reported that PC3 cells can express a transcriptionally inactive form of AR; although this was found in 2D cultures, a format in which AR protein expression was not observed, despite use of similar methodologies. Whilst many of the well characterised effects of AR in PCa cells are reliant on the genomic effects which involve the transcription of target genes, non-genomic effects of androgens also influence cell behaviour. These include the activation of kinase cascades and cytoskeletal rearrangement which can stimulate cell motility (Hatzoglou et al. 2005; Kampa et al. 2002; Papakonstanti et al. 2003). Perhaps a role for a transcriptionally inactive AR has been suggested by the findings reported here, although further investigations are required to determine the regulation of AR-target genes, other than PSA, in 3D cultures of PC3 cells.

3.3.5 DU145 Cells were AR-Negative in 2D and 3D Culture

It is interesting to note that whilst PC3 cells re-expressed AR in 3D culture, DU145 cells did not. The emergence of AR protein expression in PC3 cells and not in DU145 could perhaps be due to the absence of necessary co-activators in DU145 cells that are required to support AR expression (Litvinov et al. 2006). Whilst PC3 cells retain the expression of co-regulators required for AR activity,
DU145 cells lack this feature (Litvinov et al. 2006). The presence of co-regulators in PC3 cells may have contributed to the ability of these cells to endogenously express AR in 3D culture. Another possible mechanism through which the induction of AR expression in DU145 cells may have been prevented was their retention of phosphatase and tensin homolog (PTEN). PTEN is a known negative regulator of the AR pathway that is expressed in DU145 cells, however in PC3 or LNCaP cells it is lost (Heinlein et al. 2004). Therefore, it is possible that the presence of PTEN in DU145 cells may act to inhibit the endogenous expression of AR.

### 3.3.6 Differences in AR regulation Observed Between PC3 and LNCaP Cell Lines

The regulation of AR protein expression was found to differ between PC3 and LNCaP cells. In the PC3 cell line, inhibition of the Src signalling pathway reduced AR protein levels, an effect that was not observed in LNCaP cells. This indicates that Src activity can regulate AR protein levels in PC3, and not in LNCaP cells. The DHT-induced nuclear translocation of AR in PC3 cells was also inhibited after inhibition of Src using PP2, which indicated that Src may regulate the translocation response of AR to DHT. Src is a non-receptor tyrosine kinase that can be activated by steroids to promote cell cycle progression, cell growth, survival, migration and differentiation (Kim et al. 2009; Migliaccio 2011), in addition to promoting tumour progression and metastasis (Summy 2003).

Previously, a constitutive association between Src and AR was found to drive proliferation in an androgen-insensitive, high-passage derivative of the LNCaP cell line (LNCaP-HP; Unni 2004). Expanding on these findings that were reported by Unni and colleagues (2004), our study indicates that Src can exert a level of regulation over the AR pathway in androgen-insensitive PCa cells.

It is known that Src can function upstream of the MAPK pathway (Unni 2004). Inhibition of the MAPK pathway also resulted in inhibition of AR protein expression in PC3 cells, indicating that the Src/MAPK pathway participates in regulation of AR protein in PC3 cells. This was consistent with regulation of AR observed in the androgen-insensitive LNCaP-HP cells by the Src/MAPK pathway (Unni 2004). These data provide further evidence for the differential regulation of AR between androgen-sensitive and androgen-insensitive PCa cells.
Whilst the regulation of AR by the Src/MAPK pathway was a point of divergence between the PC3 and LNCaP cell lines, the effect of rapamycin on AR protein expression was consistent between both cell lines. Inhibition of the mTOR pathway with rapamycin resulted in an up-regulation of AR protein in both PC3 and LNCaP cells. Previously, studies in LNCaP cells have shown mTOR protein expression to inhibit AR at the level of transcription and translation (Cinar et al. 2005; Wang et al. 2008b). Here, the induction of AR protein expression in LNCaP cells upon inhibition of the mTOR pathway was consistent with that observation. Furthermore, the similar results observed in PC3 cells indicate that this regulation also extends to androgen-insensitive PCa cells.

Further studies are required, however, to determine whether the modulation of AR protein expression by rapamycin was linked to a stress response in these cultures. The mTOR pathway can regulate the growth of both PCa cells and normal prostate epithelial cells (Cao 2006; Cinar et al. 2005; Gao 2003; Ghosh 2005; Kremer 2006; Wu 2005). Here, the health of spheroids treated with rapamycin appeared to be adversely affected; their morphology showed nuclear enlargement and a shrunken cytoplasm, consistent with an apoptotic response (Jia et al. 2012). Further investigations are required to confirm the impact of rapamycin on cell viability and to determine the relevance of this regulation on the AR pathway.

Collectively, these results suggest that interactions of tumour cells with the ECM can exert an influence over AR protein expression in PCa cell lines. The AR expressed in androgen-insensitive PC3 cells cultured in 3D can display aspects of differential regulation and activity when compared to the androgen-sensitive LNCaP cell line. The consequence of AR protein re-expression in 3D cultures of PC3 cells requires further investigation, to determine the potential implications of these findings.
4 CHAPTER FOUR: REGULATION OF CXCR4 IN PCA
Experimental results from this chapter were included in a published paper, entitled “In vivo biomarkers are preserved in 3D cultures of Prostate Cancer”. Windus, L.C. Kiss, D.L. Glover, T. Avery, V.M. Experimental Cell Research. July 2012. 318: 2507-2519.

4.1 Introduction

The expressions of chemokines and their receptors are enhanced in the tumour tissues of various cancer types, providing an advantage to the growth, survival, and metastatic spread of the tumour cells (Garin 2011). Androgens may contribute to the progression of PCa by impacting on regulation of the chemokine network in tumour cells. Of particular interest is the androgenic regulation of the chemokine receptor CXCR4, due to this receptor’s role in the invasion and progression of PCa (Frigo 2009; Ngan 2009). The expression of CXCR4 in the prostate was established to be specific to malignant tissue, and expression levels correlated with more invasive tumour grade in PCa patients (Sun 2003; Wang et al. 2006). The CXCR4 ligand, SDF-1α, was highly expressed in areas such as the bone marrow, and has been shown to stimulate the migration of cancer cells to aid in the metastatic process (Wang et al. 2006).

Regulation of CXCR4 therefore represents a mechanism through which the androgen hormones can promote the progression of PCa. In the LNCaP PCa cell line, treatment with the synthetic androgen, R1881, induced the expression of CXCR4 at an mRNA and protein level (Frigo 2009; Ngan 2009). Furthermore, androgens enhanced the function of CXCR4 by promoting cell migration towards SDF-1α (Frigo 2009). This effect was mediated through the AR target gene Krüppel-like factor 5 (KLF5), a transcription factor which binds to the CXCR4 promoter and stimulates transcription of the CXCR4 gene (Frigo 2009). More recent findings have demonstrated that the AR target-gene, TMPRSS2, regulated CXCR4 expression in a different manner. The fusion protein TMPRSS2-ERG is the product of a common gene rearrangement, shown to occur in up to 50% of PCa cohorts (Park 2010b). Generated by fusion of the known oncogene ERG with the TMPRSS2 gene, TMPRSS2-ERG was found to bind directly to the CXCR4 promoter in VCap cells, mediating the up-regulation of CXCR4 expression in response to androgens (Cai 2010). These findings demonstrated that AR-regulated
genes can mediate CXCR4 expression and function in the androgen-sensitive PCa cells.

Here, the regulation of CXCR4 by androgens was investigated in androgen-sensitive and androgen-insensitive PCa cell lines grown in 2D and 3D culturing environments. In 3D culture only, up-regulation of CXCR4 protein was observed in response to DHT in PC3 cells. This is the first report, to the author's knowledge, to show that androgens were able to regulate CXCR4 via endogenous AR expression in an androgen-insensitive PCa cell line. The lack of PSA production following androgen stimulation of AR in PC3 cells was suggestive of limited AR transcriptional activity with respect to PSA in this cell line. Although a variety of factors may be influencing this activity in PC3 cells, androgenic regulation of CXCR4 in this cell line may have been mediated by specific signalling pathways in PC3 cells following AR stimulation, as opposed to the genomic regulation of CXCR4 which was previously reported in LNCaP cells via KLF5 activity (Frigo 2009).
4.2 Results

4.2.1 CXCR4 Regulation in Androgen-Sensitive LNCaP Cells

As reported in chapter 3, LNCaP cells were androgen-responsive and displayed androgen-sensitive cell growth (Fig. 3.2). Treatment of LNCaP cells with androgens was previously found to up-regulate CXCR4 expression by Frigo and colleagues (2009). To serve as a basis for comparison against studies undertaken in androgen-insensitive cell lines, this observation was firstly confirmed in LNCaP cells.

4.2.1.1 The Expression and Function of CXCR4 Protein in 2D Culture

Western blots were performed on 2D cultures of LNCaP cells and probed for CXCR4 protein expression, which revealed consistent expression in the absence and presence of SDF-1α (Fig. 4.1 A). Immunocytochemistry studies were performed using an anti-CXCR4 antibody, revealing an internalisation of the receptor in response to SDF-1α treatment (Fig. 4.1 B-C). Whilst CXCR4 in untreated cultures was predominantly observed at the cell membrane (Fig. 4.1 B-B’), treatment with SDF-1α (30 ng/ml; 40 minutes) resulted in the appearance of punctate structures in the cytoplasm, consistent with ligand-induced endocytosis (Fig. 4.1 C-C’). This receptor internalisation response was confirmed using Acapella® software by script-based image analysis of confocal images, though which the intensity of CXCR4 fluorescent signal in the cytoplasmic region was quantified before and after SDF-1α treatment (Fig. 4.1 D). A significant increase in cytoplasmic CXCR4 was observed in SDF-1α-treated samples when compared to controls (Fig. 4.1 D), consistent with internalisation of CXCR4 from the cell membrane.
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Figure 4.1 The response of CXCR4 to SDF-1α in LNCaP cells.

Expression of CXCR4 protein was confirmed via Western blot (A), demonstrating expression to be maintained in the presence of SDF-1α (50 ng/ml; 24 hours). Density was calculated in relation to the β-actin loading control. Using an anti-CXCR4 antibody, immunocytochemical staining for CXCR4 (shown in green) demonstrated that CXCR4 was localised predominantly to the cell membrane in controls (B, magnified image in B’). A re-location of CXCR4 into punctate vesicular structures in the cytoplasm after treatment with SDF-1α (30 ng/ml; 40 minutes) was consistent with receptor internalisation (C, magnified image in C’). Arrow indicates vesicles. Scale bar = 60 μm. The CXCR4 internalisation response was confirmed by script-based image analysis using Acapella® software (D). The fluorescence intensity of the CXCR4 staining in the cytoplasmic region of the cell was quantified over a time course of SDF-1α treatment on confocal images. A statistically significant increase in cytoplasmic CXCR4 was demonstrated after 40-60 minutes of ligand stimulation. ***p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.1.1 SDF-1α-Induced Signalling

Downstream of CXCR4 activation, phosphorylation of the PI3K/Akt and MAPK pathways have been reported in PCa cell lines (Wang et al. 2006; Wang et al. 2005a). To investigate this response in LNCaP cells, whole cell lysates were probed for phospho-Akt and phospho-ERK via Western blot. No relative increase in phospho-Akt (Fig. 4.2 A) or phospho-ERK 1/2 (Fig. 4.2 B) was observed after stimulation with SDF-1α (100 ng/ml) for up to 60 minutes. These results are consistent with a lack of SDF-1α-induced signalling through the PI3K/Akt and MAPK pathways in LNCaP cells.

![Figure 4.2](image)

*Figure 4.2 The effect of SDF-1α stimulation on intracellular signalling in LNCaP cells.*

Cells were serum-starved for 16 hours prior to application of SDF-1α (100 ng/ml) for 5-60 minutes. Whole cell lysates were analysed via Western blot for phospho-Akt and total Akt (A), or phospho-ERK 1/2 and total ERK 1/2 (B). No phosphorylation response was observed in these pathways in response to SDF-1α for up to 60 minutes. Densitometry was expressed relative to the appropriate loading control for each sample (total Akt, total ERK 1 or total ERK 2). In the case of total Akt, two bands were consistently produced in LNCaP experiments (n=3). The major upper band in the total Akt study was used for quantification, because this band specifically corresponded to the size of the band for phospho-Akt which was evident on re-probing. Results shown are representative of four separate experiments, with densitometry performed on representative data.
4.2.1.1.2 Cell Migration Response to SDF-1α

Consistent with its role as a chemokine receptor, activation of CXCR4 is typically followed by a cell migration response in PCa cells (Busillo et al. 2007; Wang et al. 2006). When LNCaP cells were seeded in Transwell® chamber cell migration assays, however, no cell migration response was observed in response to SDF-1α (200 ng/ml) in this cell line (Fig. 4.3 A-C). The mean number of migrated cells present on the underside of the Transwell® inserts was consistent between controls and SDF-1α-treated cultures (Fig. 4.3 A-C). Re-optimisation of assay conditions, including assay incubation time (6 hours to 24 hours), cell seeding density (10,000-150,000 cells/well) and SDF-1α concentration (200-500 ng/ml) did not result in a measurable cell migration response. These results indicate that, whilst LNCaP cells expressed ligand-responsive CXCR4, no SDF-1α-induced LNCaP cell migration response was observed.

![Figure 4.3 The effect of SDF-1α on LNCaP cell migration.](image)

LNCaP cells were serum-starved for 16 hours prior to seeding into the upper chamber of Transwell® cell migration assays (80,000 cells/insert). SFM alone, or containing SDF-1α (200 ng/ml), were added to the lower chamber and assays were incubated at 37°C for 6 hours, prior to fixation and staining with crystal violet. A representative brightfield image of stained cells on the underside of the Transwell® insert was shown for controls (A) and SDF-1α-treated wells (B). Scale bar = 600 µm. Cell migration was quantified by manually counting the number of migrated cells per field (5 fields per insert; 40x objective) (C). Data was analysed using a paired t-test, and the results were not statistically significant. Error bars denote S.E.M. Results shown are representative of three separate experiments.
4.2.1.2 Expression and Function of CXCR4 Protein in 3D Culture

Next, 3D culturing methods were used to study CXCR4 protein expression in LNCaP cells. A direct comparison between 2D and 3D cultures revealed consistent CXCR4 expression levels via Western blot analysis (Fig. 4.4 A). When analysed by immunocytochemistry using an anti-CXCR4 antibody, CXCR4 was located on the periphery of the spheroid and localised predominantly to the cell membrane (Fig. 4.4 B-B’). Treatment with SDF-1α (30 ng/ml, 40 minutes) resulted in movement of the receptor into the cell cytoplasm (Fig. 4.4 C-C’). The receptor internalisation response was further confirmed by script-based image analysis using Acapella® software, which demonstrated a statistically significant increase in mean cytoplasmic CXCR4 intensity after treatment with SDF-1α, over 40-60 minute period (Fig. 4.4 D).
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Figure 4.4 LNCaP cells expressed ligand-responsive CXCR4 in 3D culture.

Expression of CXCR4 protein in 2D and 3D cell cultures was confirmed via Western blot, demonstrating a similar expression between culture conditions (A). Density was calculated in relation to the β-actin loading control. The response of CXCR4 to stimulation with SDF-1α was assessed by immunocytochemistry using an anti-CXCR4 antibody (B-C). In untreated cultures, CXCR4 (shown in green) was observed to reside predominantly at the cell membrane on the periphery of spheroids (B-B’). Treatment with SDF-1α (30 ng/ml; 40 minutes) increased the cytoplasmic content of CXCR4 (C-C’). Arrow indicates cytoplasmic CXCR4. Scale bar = 60 µm. The receptor internalisation response to SDF-1α treatment over time was subsequently confirmed using script-based image analysis (D). The fluorescent intensity of CXCR4 staining in the cytoplasmic region was quantified using Acapella® software analysis on confocal images. A statistically significant increase in cytoplasmic CXCR4 was observed after 40-60 minutes of ligand treatment. **p<0.01 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.1.3 The Effect of Androgens on CXCR4 Regulation in 2D Culture

The AR pathway not only regulates prostate cell growth and protein secretion in PCa (Falkenstein 2000; Kaarbo et al. 2007), it also impacts on pro-migratory chemokine receptors, which have been established as key mediators of PCa metastasis (Akashi et al. 2006; Frigo 2009). The endogenous co-expression of CXCR4 and AR in the LNCaP cell line permitted an investigation into AR-mediated regulation of CXCR4 at a protein level. Previously, Frigo and colleagues (2009) have reported an AR-mediated up-regulation of CXCR4 expression in response to androgens in LNCaP cells, which enhanced LNCaP cell migration towards an SDF-1α gradient.

Here, an enhancement of CXCR4 protein expression was observed in response to androgens in LNCaP cells cultured in 2D (Fig. 4.5). As demonstrated by Western blot analysis, CXCR4 protein expression was up-regulated in response to 5-10 nM DHT for 30 hours (Fig. 4.5 A). This result was further confirmed via immunocytochemistry using an anti-CXCR4 antibody (Fig. 4.5 B-C). In comparison to control cells (Fig. 4.5 B), cultures treated with 10 nM DHT for 30 hours displayed enhanced CXCR4 protein expression (shown in green), as indicated by an enhanced fluorescent signal intensity (Fig. 4.5 C). This observation was further confirmed using script-based image analysis to quantify CXCR4 intensity at the cell membrane, demonstrating the CXCR4 intensity values were significantly up-regulated in cultures treated with DHT (Fig. 4.5 D).
Figure 4.5 Up-regulation of CXCR4 protein by androgens in 2D cultures of LNCaP cells.

LNCaP cells were cultured in 2D for 10 days prior to the addition of SFM alone or containing DHT (10 nM) for 30 hours. Samples were collected and lysed for Western blot analysis for CXCR4 protein expression, using β-actin as a loading control (A). CXCR4 protein expression was also assessed using an anti-CXCR4 antibody in immunocytochemistry (B-C). When compared to controls (B-B’), the expression of CXCR4 was enhanced in response to DHT treatment (C-C’). Scale bar = 60µm. Acapella® software was used to perform script-based image analysis of CXCR4 fluorescence intensity on confocal images (D). A statistically significant up-regulation of CXCR4 protein was observed in cultures treated with DHT (5-10 nM) when compared to controls. *p<0.05, **p<0.01 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.1.4 The Effect of Androgens on CXCR4 Regulation in 3D Culture

Consistent with observations in 2D culture, 3D cultures of LNCaP also displayed an enhanced level of CXCR4 protein in response to DHT treatment (Fig. 4.6 A). When compared to controls, Western blot analysis demonstrated that LNCaP cells cultured in the presence of DHT (5-10 nM) for 30 hours displayed a higher level of CXCR4 protein expression (Fig. 4.6 A). This observation was also confirmed via immunocytochemistry using an anti-CXCR4 antibody (Fig. 4.6 B-C), comparing the intensity of CXCR4 staining in controls (Fig. 4.6 B-B’) to the increased fluorescent signal observed in DHT-treated cultures (10 nM; Fig. 4.6 C-C’). To confirm this observation, CXCR4 fluorescence intensity was quantified at the cell membrane using script-based Acapella® software image analysis. In comparison to controls, a statistically significant up-regulation of CXCR4 signal intensity at the cell membrane was observed in response to 5-10 nM of DHT (Fig. 4.6 D).
Figure 4.6 Up-regulation of CXCR4 protein by androgens in 3D cultures of LNCaP cells.

LNCaP cells were cultured in 3D for 10 days, prior to the addition of SFM in the absence or presence of DHT (10 nM) for 30 hours. Samples were collected for analysis via Western blot for CXCR4 protein expression (A). Density was calculated in relation to the β-actin loading control. Immunocytochemistry was performed using an anti-CXCR4 antibody to analyse CXCR4 protein expression. When compared to controls (B-B’), an enhanced level of CXCR4 protein (shown in green) was observed in cultures treated with DHT (10 nM) (C-C’). CellMask™ Blue is shown in blue and DRAQ5™ nuclear stain in red. Scale bar = 60 µm. Acapella® software was employed to analyse the intensity of CXCR4 protein expression in confocal images, showing a significant increase in CXCR4 after treatment with 5-10nM DHT (D). *p<0.05, **p<0.01 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.1.4.1 The Role of AR in CXCR4 Regulation in LNCaP 3D Cultures

To confirm that AR activity was required for CXCR4 protein up-regulation in response to DHT treatment, the AR antagonist bicalutamide was incubated with 3D LNCaP cultures in the presence of DHT (Fig. 4.7). As observed by Western blot, bicalutamide prevented the DHT-induced enhancement of CXCR4 protein expression (Fig. 4.7). The efficiency of bicalutamide in preventing this response demonstrated a requirement for AR activity in mediating CXCR4 protein up-regulation, consistent with the results of Frigo and colleagues (2009).

![Western blot image](image)

**Figure 4.7** Up-regulation of CXCR4 protein by androgens in 3D cultures of LNCaP cells was mediated by AR activity.

LNCaP cells were cultured in 3D for 10 days prior to treatment with vehicle (control), DHT (10 nM) and/or bicalutamide (5 µM) for 30 hours. Samples were collected and analysed for CXCR4 protein expression via Western blot. The enhanced CXCR4 protein expression observed in response to DHT was prevented by co-incubation with bicalutamide. Density was calculated in relation to the β-actin loading control. Results shown are representative of three separate experiments, with densitometry performed on representative data.

The use of cell migration assays to measure the effect of androgens on CXCR4-mediated cell migration was not feasible due to the lack of LNCaP cell migration observed in response to SDF-1α. However, to further characterise the regulation of CXCR4 by androgens, the role of AR-induced intracellular pathways in the regulation of CXCR4 protein expression was investigated.
4.2.1.4.2 Regulation of CXCR4 by Intracellular Signalling Pathways in LNCaP

The stimulation of prostate cells with androgens is followed by activation of intracellular signalling pathways, including PI3K/Akt and MAPK, resulting in the transcription of target genes (Kaarbo et al. 2007). Here, various signalling pathway inhibitors were employed to assess androgen regulation of CXCR4 protein expression in LNCaP cells, to serve as a basis for comparison in subsequent studies of androgen-insensitive PCa cells.

To this aim, wortmannin (PI3K/Akt inhibitor), rapamycin (mTOR inhibitor), PP2 (Src inhibitor) its inactive analogue PP3, or PD98059 (MAPK inhibitor) were applied to established LNCaP spheroids after 9 days in culture. The following day, culture medium was replenished containing fresh inhibitors, in the absence or presence of DHT (10 nM), and incubated for 30 hours. Following treatment, cultures were assessed for CXCR4 protein expression via Western blot (Fig. 4.8 A) or immunocytochemistry using an anti-CXCR4 antibody (Fig. 4.8 B-C). Western blot analysis of control cultures demonstrated that incubation with DHT induced CXCR4 protein up-regulation, a response which was maintained in the presence of the Src kinase inhibitor PP2, its inactive analogue PP3, the MAPK inhibitor PD98059 or the mTOR inhibitor rapamycin (Fig. 4.8 A). Incubation with the PI3K/Akt inhibitor wortmannin, however, reduced basal CXCR4 protein expression in LNCaP cells (Fig. 4.8 A). This response was confirmed via immunocytochemistry; when compared to the vehicle control (Fig. 4.8 B-B’), CXCR4 was down-regulated in cultures pre-treated with wortmannin (Fig. 4.8 C-C’). These results indicate that the PI3K/Akt signalling pathway can regulate CXCR4 protein expression in this cell line. However, a more specific inhibitor of the PI3K/Akt pathway, such as LY294002, is required to confirm the role of this signalling pathway in the regulation of CXCR4 protein expression.
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Figure 4.8 The effect of signalling pathway inhibitors on androgen regulation of CXCR4 in LNCaP cells.

Cells were cultured in 3D for 9 days, pre-treated with signalling pathway inhibitors including Src kinase inhibitor PP2 (5 µM), its inactive analogue PP3 (5 µM), mTOR inhibitor rapamycin (1 µM), PI3K/Akt inhibitor wortmannin (20 µM) or MAPK inhibitor PD98059 (10 µM) or DMSO vehicle (0.4%) for 24 hours, before addition of fresh inhibitors in the presence or absence of DHT (10 nM) for a further 30 hours. Samples were collected and lysed prior to analysis for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. The effects of DHT on CXCR4 protein expression after treatment with DMSO vehicle (B-B’) and wortmannin (C-C’) were also assessed by immunocytochemistry using an anti-CXCR4 antibody. CXCR4 is shown in green, phalloidin staining of F-actin in red, and DRAQ5™ staining of nuclei in blue. Scale bar = 60 µm. Results shown are representative of three separate experiments, with densitometry performed on representative data.

Thus far, these results have demonstrated that DHT treatment resulted in an up-regulation of CXCR4 protein in both 2D and 3D cultures of LNCaP cells. Furthermore, DHT-induced up-regulation of CXCR4 protein in 3D cultures of LNCaP was mediated through AR activity, and occurred independently of activity in the Src/MAPK and mTOR signalling pathways.
4.2.2 CXCR4 Regulation in Androgen-Insensitive PC3 Cells

The more invasive, androgen-insensitive PC3 cells were then used to investigate the regulation of CXCR4 protein expression, comparing between in 2D and 3D culture conditions.

4.2.2.1 The Expression and Function of CXCR4 Protein in PC3 Cells

The expression and function of endogenous CXCR4 protein was assessed in the PC3 cell line using 2D culture. As expected, PC3 cells expressed CXCR4 in the absence and presence of its ligand SDF-1α (50 ng/ml; 24 hours), as shown via Western blot (Fig. 4.9 A). When examined via immunocytochemistry using an anti-CXCR4 antibody, CXCR4 (shown in green) was localised to the cell membrane (Fig. 4.9 B-B’). Upon stimulation with SDF-1α, the emergence of punctuate vesicular structures within the cell cytoplasm were indicative of receptor internalisation (arrow, Fig. 4.9 C-C’). The translocation of the receptor from the cell membrane to the cytoplasm was reflected in the increased cytoplasmic intensity of CXCR4 after treatment with SDF-1α, as quantified through image analysis using Acapella® software (Fig. 4.9 D).
Figure 4.9 CXCR4 in PC3 cells was responsive to SDF-1α stimulation.

PC3 cells were cultured in 2D for 9 days, serum-starved in the presence or absence of SDF-1α (30 ng/ml, 24 hours) prior to collection. Whole cell lysates were analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. Immunocytochemistry was performed using an anti-CXCR4 antibody (B-C) to examine receptor localisation. CXCR4 protein expression (green fluorescence) in controls was located predominantly on the cell membrane (B, magnified image in B’). Cells stimulated with SDF-1α (100 ng/ml, 40 minutes) contained punctate vesicular structures (C, magnified image in C’), a feature which was consistent with a receptor internalisation response. Arrow indicates vesicular structures. Scale bar = 60µm. The CXCR4 internalisation response was confirmed through script-based image analysis using Acapella® software (D). The mean fluorescence intensity of CXCR4 staining in the cytoplasm was quantified, showing a statistically significant increase in cytoplasmic CXCR4 signal after 40-60 minutes of SDF-1α stimulation. ***p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.2.1.1 SDF-1α-Induced Signalling in PC3 Cells

Following ligand-induced activation of the receptor, CXCR4 can elicit signalling through the MAPK and PI3K/Akt pathways (Busillo et al. 2007; Sun 2003; Wang et al. 2005a). In 2D cultures of PC3 cells, Akt phosphorylation was observed after 30-60 minutes of SDF-1α stimulation (Fig. 4.10 A). This response was not inhibited by pre-incubation with the CXCR4 antagonist AMD3100, which indicated that phosphorylation of this pathway was not dependent on CXCR4 activity. When activation of the MAPK signalling pathway was assessed, no phosphorylation of ERK 1/2 was observed in response to SDF-1α treatment (Fig. 4.10 B).

Figure 4.10 SDF-1α-induced signalling in PC3 cells.

Cells were cultured for 24 hours, prior to application of SFM in the absence or presence of AMD3100 (5 µg/ml) for 16 hours. The following day, cells were stimulated with SDF-1α (100 ng/ml) for 5-60 minutes prior to lysis and sample collection. Whole cell lysates were analysed via Western blot for phospho-Akt and total Akt (A), or phospho-ERK 1/2 and total ERK/12 (B). Densitometry was expressed as a relative density of the loading control (total Akt or total ERK/12) for each sample. Results shown are representative of four separate experiments, with densitometry performed on representative data.
4.2.2.1 PC3 Cell Migration Response to SDF-1α

Subsequent to intracellular signalling, SDF-1α-induced activation of CXCR4 stimulates cell migration in PCa cells (Busillo et al. 2007; Wang et al. 2005a). After establishing the optimal seeding density for the Transwell® cell migration assay (Fig. 4.11), PC3 cell migration was assessed (Fig. 4.12). When compared with controls (Fig. 4.12 A), an increase in the numbers of PC3 cells migrating to the underside of the Transwell® insert were observed in the presence of SDF-1α (Fig. 4.12 B). Quantified data generated from mean cell counts demonstrated a statistically significant cell migration response to SDF-1α in PC3 cells, which was inhibited by pre-incubation with AMD3100 (Fig. 4.12 C).

![Figure 4.11 Optimisation of PC3 cell seeding density for cell migration assays.](image)

PC3 cells were seeded at densities of 10,000-30,000 cells/well into Transwell® cell migration assays to optimise the migration response to SDF-1α. Cells were seeded into the upper chamber in serum-free media, whilst the lower chamber contained SFM alone or SDF-1α (200 ng/ml) as a chemoattractant. After a 6 hours of incubation at 37°C, Transwell® inserts were fixed and stained with crystal violet. Quantification of migration was obtained by manual counting of stained cells on the underside of Transwell® inserts. Five fields of view per insert were counted per insert (40x objective; Eclipse TS100 microscope (Nikon). **p<0.01 paired t-test. Error bars denote S.E.M. Results shown are representative of three separate cell populations, tested in duplicate.
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Figure 4.12 CXCR4-mediated migration towards SDF-1α in PC3 cells.
PC3 cells were cultured for 24 hours prior to incubation in serum-free media, in the presence or absence of the CXCR4 antagonist AMD3100 (5µg/ml) for 16 hours. The next day, cells were seeded in Transwell® chamber assays at a density of 30,000 cells/well. Lower chambers contained either SFM alone (control) or SDF-1α (200ng/ml) as a chemoattractant. Assays were incubated for 6 hours at 37°C, prior to fixation and staining of inserts with crystal violet. Migrated cells stained with crystal violet on the underside of the Transwell® insert are shown for control wells (A) and SDF-1α treated wells (B). Scale bar = 600 µm. The cell migration response was quantified through manual cell counting (five fields of view per insert; 40x objective) (C). *p<0.05, **p<0.01 paired t-test. Error bars denote S.E.M. Results shown are representative of three separate experiments.

4.2.2.2 The Effect of 3D Culture on CXCR4 Protein Expression in PC3 Cells
The regulation of CXCR4 was then investigated in 3D cultures of PC3 cells. A direct comparison of CXCR4 protein levels between 2D and 3D cultures revealed an enhanced protein expression of the receptor in 3D cultures, as detected via Western blot (Fig. 4.13 A). Three-dimensional cultures of PC3 cells were then investigated for the response of CXCR4 to ligand stimulation. Whilst the addition of SDF-1α to 3D cultures did not impact on total protein expression of CXCR4 (Fig. 4.13 A), analysis of cultures by immunocytochemistry using an anti-CXCR4 antibody demonstrated a receptor internalisation response to SDF-1α (30 ng/ml, 20 minutes) (Fig. 4.13 B-C). Unstimulated cultures expressed CXCR4 (shown in green) predominantly on the cell membrane (Fig. 4.13 B-B’), and cultures stimulated with SDF-1α showed an increase in cytoplasmic CXCR4 localisation (Fig. 4.13 C-C’). A statistically significant up-regulation of CXCR4 cytoplasmic content was observed after 40-60 minutes of SDF-1α stimulation, as quantified by script-based image analysis for CXCR4 fluorescence intensity in the cytoplasm (Fig. 4.13 D).
Figure 4.13 3D cultures of PC3 cells displayed enhanced protein expression of ligand-responsive CXCR4.

Cells were cultured in 3D for 10 days prior to serum-starvation in the presence or absence of SDF-1α (30 ng/ml) for 24 hours. Samples were collected and analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. Immunocytochemistry was also performed on PC3 cultures using an anti-CXCR4 antibody to examine receptor localisation. In control cultures, CXCR4 (shown in green) was located predominantly to the cell membrane (B-B’). In response to stimulation with SDF-1α (30 ng/ml, 40 minutes), a translocation of CXCR4 into the cytoplasm was observed (C-C’). Scale bar = 60 μm. Results are representative of three separate experiments. Receptor internalisation in response to SDF-1α was quantified through script-based image analysis using Acapella® software (D). The intensity of CXCR4 fluorescence in the cytoplasmic region of confocal images was quantified, showing a statistically significant increase CXCR4 cytoplasmic intensity after treatment with SDF-1α for 40-60 minutes, ***p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.2.3 CXCR4 Regulation by Androgens in PC3 Cells

Subsequent to these findings, the regulation of CXCR4 in 3D cultures of PC3 cells was subject to further characterisation in relation to androgens and the AR. Although regulation of CXCR4 by androgens has been reported in LNCaP cells (Frigo 2009), there are no reports of this regulation occurring endogenously in androgen-insensitive PCa cell lines.

As expected, the addition of DHT (5-10 nM; 30 hours) to AR-negative PC3 cells in 2D culture did not impact on CXCR4 protein expression, as demonstrated by Western blot analysis (Fig. 4.14 A). This was further confirmed via immunocytochemistry using an anti-CXCR4 antibody, which demonstrated consistent CXCR4 protein expression and distribution (shown in green) between control (Fig. 4.14 B-B’) and DHT-treated cultures (Fig. 4.14 C-C’).
Figure 4.14 The protein expression of CXCR4 in 2D cultures of PC3 cells was unaffected by androgens.

PC3 cells were cultured in 2D for 10 days. On the final day, cells were incubated with SFM alone or containing DHT (10 nM) for 30 hours prior to sample collection. Cells were lysed and analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. Using an anti-CXCR4 antibody, the expression of CXCR4 protein (shown in green) in both control (B-B’) and DHT-treated (C-C’) cultures was observed to be localised as diffuse staining in the cytoplasm, towards the cell membrane. CellMask™ Blue is shown in blue, and DRAQ5™ nuclear stain in red. Scale bar = 60 µm. Results shown are representative of three separate experiments, with densitometry performed on representative data.
The emergence of AR protein expression in 3D cultures of PC3 cells permitted an investigation of CXCR4 regulation by androgens in this androgen-insensitive PCa cell line. PC3 cells were cultured in 3D for 10 days, prior to treatment with DHT (10 nM) for 30 hours. Whole cell lysates were then probed for CXCR4 protein expression via Western blot (Fig. 4.15 A). Interestingly, an up-regulation of CXCR4 protein was observed under these 3D conditions (Fig. 4.15 A), an effect which was not observed in AR-negative 2D cultures of PC3 cells (Fig. 4.14 A). Immunocytochemistry revealed an enhanced protein expression of CXCR4 in response to treatment with DHT when compared to controls (Fig. 4.15 B, C). This response was further confirmed by image analysis of mean CXCR4 intensity at the cell membrane (Fig. 4.15 D), which was significantly enhanced in response to DHT treatment. Western blots demonstrated that co-incubation with bicalutamide prevented this up-regulation of CXCR4 protein by androgens (Fig. 4.15 E). These findings demonstrated that AR and androgens can modulate CXCR4 protein expression in androgen-insensitive PC3 cells.
Figure 4.15 Up-regulation of CXCR4 protein in response to androgens in 3D cultures of PC3 cells.

PC3 cells were cultured in 3D for 10 days. On the final day, cells were incubated with SFM alone, or containing DHT (10 nM), for 30 hours prior to sample collection. Cells were lysed and analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. Immunocytochemical analysis of CXCR4 protein expression was also performed using an anti-CXCR4 antibody (B-E). In control cultures, the basal level of CXCR4 protein expression (shown in green) was demonstrated in both a single confocal slice (B-B’) and a reconstructed 3D z-stack (C-C’). After treatment with DHT (10 nM, 30 hours), an up-regulation of CXCR4 protein was observed, as demonstrated in both the confocal slice image (D-D’) and the reconstructed z-stack (E-E’). CellMask™ Blue is shown in blue, and DRAQ5™ nuclear stain in red. Scale bar = 60 µm. The up-regulation of CXCR4 in response to DHT was also confirmed by script-based image analysis of confocal images, using Acapella® software (D). A statistically significant increase in the mean CXCR4 signal in the cell membrane region was observed in response to DHT treatment (5-10 nM). *p<0.05, **p<0.01 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40 spheroids. The effect of bicalutamide on DHT-induced CXCR4 protein up-regulation was then investigated by Western blot analysis (E). PC3 cells cultured in 3D were incubated in serum-free media containing DHT (10 nM), bicalutamide (5 µM), or a combination of DHT and bicalutamide, for 30 hours. Whole cell lysates were probed for CXCR4 protein expression (E). Bicalutamide was observed to prevent the enhancement of CXCR4 protein levels in response to DHT, demonstrating that AR activity was required for the effect on CXCR4. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.2.3.1 Signalling Pathways Regulating CXCR4 Protein Expression in PC3

To further characterise the pathways mediating this regulation of CXCR4 protein expression in 3D cultures of PC3 cells, signalling pathway inhibitors were employed. Inhibitors targeting the Src (PP2), PI3K (wortmannin), mTOR (rapamycin) and MAPK (PD98059) pathways were used to study the effect on CXCR4 regulation in PC3 cells. Samples analysed via Western blot demonstrated that DHT-induced up-regulation of CXCR4 protein was unaffected by treatment with wortmannin or PP3 (Fig. 4.16 A). Incubation with the Src inhibitor PP2 and the MAPK inhibitor PD98059, however, prevented DHT-induced up-regulation of CXCR4 protein (Fig. 4.16 A). Immunocytochemistry studies demonstrated that the CXCR4 up-regulation observed in response to androgens in uninhibited cultures (Fig. 4.16 B-B’) was prevented in cultures pre-treated with PP2 (Fig. 4.16 C-C’) or PD98059 (Fig. 4.16 D-D’). This indicated that activity through the Src and MAPK signalling pathways were required for DHT-induced up-regulation of CXCR4 protein in PC3 cells.

Treatment of PC3 cultures with rapamycin enhanced the basal protein expression of CXCR4, as shown via Western blot (Fig. 4.16 A) and immunocytochemistry (Fig. 4.16 E-E’). However, the observed nuclear enlargement and altered cell contacts within the spheroids may be indicative of a stress response in these cells after treatment with rapamycin, particularly in cultures treated with both rapamycin and DHT. These observations indicate that cell viability may be compromised under this condition, a result which confounded any interpretation of effects on CXCR4 protein expression.
Figure 4.16 The effect of signalling pathway inhibitors on androgen regulation of CXCR4 in 3D cultures of PC3 cells.

Cells were cultured in 3D for 9 days, prior to incubation with Src kinase inhibitor PP2 (5 µM), its inactive analogue PP3 (5 µM), mTOR inhibitor rapamycin (1 µM), PI3K/Akt inhibitor wortmannin (20 µM) or MAPK inhibitor PD98059 (10 µM) or DMSO vehicle (0.4%) for 24 hours. The next day, SFM containing fresh inhibitor was applied to cultures, either alone or in the presence of DHT (10 nM) for 30 hours. Samples were collected and analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. Immunocytochemical analysis was performed using an anti-CXCR4 antibody, to examine the effect of DMSO vehicle (B), PP2 (C), PD98059 (D) or rapamycin (E) on CXCR4 protein expression. CXCR4 is shown in green, phalloidin staining of F-actin in red, and Hoechst nuclear stain in blue. Treatment with PP2 and PD98059 inhibited the expression of AR protein in PC3 cells. Scale bar = 60µm. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.2.3.2 Consequences of CXCR4 Regulation in PC3 Cells

The downstream effects of androgens on CXCR4 activity were then investigated in 3D cultures of PC3 cells. Cell migration assays were employed as a measure of CXCR4 functionality, to determine whether a corresponding enhancement of CXCR4-mediated migration response could be observed in androgen-stimulated PC3 cells cultured in 3D. Cells were extracted from Matrigel and seeded in Transwell® cell migration assays. However no detectable cell migration response towards SDF-1α was observed after multiple attempts using these extracted cell populations (n=6), most likely due to compromised cell viability from the extraction process.

4.2.3 CXCR4 Regulation in Androgen-Insensitive DU145 Cells

Subsequent to investigations in PC3 cells, CXCR4 protein expression and function was analysed in the androgen-insensitive DU145 cell line.

4.2.3.1 The Expression and Function of CXCR4 Protein in DU145 Cells

Western blots of 2D DU145 cultures were probed for CXCR4 protein expression, and consistent expression was observed in the absence or presence of SDF-1α (50 ng/ml, 24 hours; Fig. 4.17 A). When investigated via immunocytochemistry using an anti-CXCR4 antibody (Fig. 4.17 B-C), a receptor internalisation response was observed. In controls, diffuse staining of CXCR4 (shown in green) was observed, located towards the cell membrane (Fig. 4.17 B-B’). Treatment with SDF-1α resulted in the appearance of punctate vesicular structures in the cytoplasm (arrow, Fig. 4.17 C-C’), consistent with ligand-induced endocytosis of CXCR4. This internalisation response was further confirmed by script-based image analysis of mean cytoplasmic CXCR4 intensity using Acapella (Fig. 4.17 D). A significant increase in cytoplasmic CXCR4 intensity was observed after treatment with SDF-1α for 40-60 minutes (Fig. 4.17 D).
Figure 4.17 Two-dimensional cultures of DU145 expressed ligand-responsive CXCR4.

Cells were cultured in 2D for 10 days, and serum-starved in the presence or absence of SDF-1α (50 ng/ml) for 24 hours. Whole cell lysates were analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. Alternatively, an anti-CXCR4 antibody was used to study receptor localisation via immunocytochemistry. In controls, CXCR4 (green fluorescence) was present as diffuse staining within the cytoplasm and on the cell membrane (B-B’). Treatment with SDF-1α (50 ng/ml; 20 minutes) resulted in the appearance of vesicular structures in the cytoplasm (C-C’), consistent with receptor internalisation. Arrow indicated punctate vesicular structures. DRAQ5™ nuclear labelling is shown in red, and Cell Mask™ Blue in blue. Scale bar = 60 µm. The intensity of CXCR4 fluorescence in the cell cytoplasm was quantified using Acapella® analysis software on confocal images (D). A statistically significant increase in cytoplasmic CXCR4 content was observed after 40-60 minutes of SDF-1α stimulation. ***p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.3.1.1 SDF-1α-Induced Signalling in DU145

In DU145 cells, the activation of PI3K/Akt and MAPK signalling pathways after SDF-1α stimulation was investigated via Western blot. SDF-1α treatment resulted in a robust phosphorylation response of Akt, observed after 30-60 minutes of stimulation (Fig. 4.18 A). Pre-treatment with the CXCR4 antagonist AMD3100 did not inhibit this response, indicating that CXCR4 was not responsible for activity through the Akt pathway (Fig. 4.18 A). Analysis of ERK 1/2 via Western blot revealed a strong phosphorylation of this pathway after 5-15 minutes of SDF-1α stimulation (Fig. 4.18 B). This activity was dependent on CXCR4 activity, as incubation with the CXCR4 antagonist AMD3100 inhibited the phosphorylation response (Fig. 4.18 B).

**Figure 4.18 SDF-1α-induced signalling in DU145 cells.**

Cells were cultured for 24 hours, and serum-starved in the presence or absence of AMD3100 (5 µg/ml) for 16 hours. The following day, cells were stimulated with SDF-1α (100 ng/ml) for 5-60 minutes, prior to collection of whole cell lysates. Samples were analysed via Western blot for phospho-Akt and total Akt (A), or phospho-ERK 1/2 and total ERK 1/2 (B). Densitometry was expressed as a relative density of the loading control (total Akt or total ERK/12) for each sample. Results shown are representative of four separate experiments, with densitometry performed on representative data.
4.2.3.1.2 The Cell Migration Response of DU145 Cells to SDF-1α

Following optimisation of DU145 seeding densities for Transwell® cell migration assays (Fig. 4.19), the ability of DU145 cells to migrate towards SDF-1α was measured (Fig. 4.20). In comparison to the basal response in controls (Fig. 4.20 A), an increase in cell migration was observed in the presence of SDF-1α (200 ng/ml) (Fig. 4.20 B). Quantification of the cell migration response demonstrated a statistically significant increase in the presence of SDF-1α, a response which was inhibited by pre-treatment with AMD3100 (Fig. 4.20 C).

![Figure 4.19 Optimisation of DU145 cell seeding density for cell migration assays.](image)

DU145 cells (30,000 to 80,000 cells/well) were tested in Transwell® cell migration assays to determine the optimal window between controls and the SDF-1α-induced migration response. Cells were seeded into the upper well in SFM, and the lower well contained either SFM alone or with SDF-1α (200 ng/ml) as a chemoattractant. After incubation for 6 hours at 37°C, inserts were fixed and stained with crystal violet. The numbers of migrated cells on the underside of the inserts were counted manually (5 fields per insert; 40x objective). A statistically significant increase in cell migration was observed in response to SDF-1α at a cell density of 80,000 cells/well; this seeding density was used in all subsequent DU145 cell migration assays. *p<0.05 paired t-test. Error bars denote S.E.M. Results shown are representative of three separate cell populations, tested in duplicate.
Figure 4.20 CXCR4-mediated migration of DU145 cells to SDF-1α.

DU145 cells were serum-starved in the presence or absence of AMD3100 (5 µg/ml) for 24 hours, prior to seeding into the upper chamber of Transwell® chamber assays at a density of 80,000 cells/well. The lower chamber contained either serum-free media (control) or SDF-1α (200 ng/ml) as a chemoattractant. Assays were incubated for 6 hours prior to fixation and staining with crystal violet. Fixed and stained cells were visualised on the underside of the Transwell® insert, and brightfield images were shown for both control wells (A) and SDF-1α treated wells (B). Scale bar = 600 µm. Counts were performed on five fields of view per Transwell® insert (40x objective, Eclipse TS100 microscope, Nikon). **p<0.01, ***p<0.001 paired t-test. Error bars denote S.E.M. Results shown are representative of three separate experiments.

4.2.3.2 CXCR4 Protein Expression in 3D Cultures of DU145 Cells

The expression of CXCR4 protein was then investigated in 3D cultures of DU145 cells. The level of CXCR4 protein expression was found to be consistent between 2D and 3D cultures as observed via Western blot (Fig. 4.21 A). In response to SDF-1α stimulation, a receptor internalisation response was visualised via immunocytochemistry using an anti-CXCR4 antibody (Fig. 4.21 B-C). In untreated cultures, CXCR4 (green fluorescence) was distributed on the periphery of spheroids, localised to both the cell membrane and, to a lesser extent, in the cytoplasm (Fig. 4.21 B-B’). Stimulation with SDF-1α enhanced the cytoplasmic localisation of CXCR4 (arrow, Fig. 4.21 C-C’), consistent with a receptor internalisation response. Script-based image analysis of the mean cytoplasmic intensity for CXCR4 staining confirmed a statistically significant increase in CXCR4 cytoplasmic intensity after 40-60 minutes of stimulation with SDF-1α (Fig. 4.21 D).
Figure 4.21  3D cultures of DU145 cells maintained ligand-responsive CXCR4 protein expression.

Cells were cultured in 2D or 3D for 10 days prior to serum-starvation in the presence or absence of SDF-1α (50 ng/ml) for 24 hours. Whole cell lysates were analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control (A). Using an anti-CXCR4 antibody, receptor localisation was studied via immunocytochemistry (B-C). In controls, CXCR4 expression (shown in green) was present as diffuse staining towards the spheroid periphery (B-B’). Treatment with SDF-1α (30 ng/ml; 20 minutes) enhanced the cytoplasmic localisation of CXCR4 (C-C’) indicating receptor internalisation in response to ligand. Arrow indicated cytoplasmic CXCR4. DRAQ5™ nuclear labelling is shown in red and CellMask™ Blue in blue. Scale bar = 60µm. The receptor internalisation response was quantified using script-based image analysis (D). Using Acapella® software to analyse confocal images, a statistically significant increase in cytoplasmic CXCR4 intensity was measured in response to SDF-1α treatment or 40-60 minutes (D). ***p<0.001 post-hoc Dunn’s Multiple Comparison test. Error bars denote S.E.M. n=35-40. Results shown are representative of three separate experiments, with densitometry performed on representative data.
4.2.3.3 The Effect of Androgens on CXCR4 Regulation in DU145 Cells

The effects of androgens on CXCR4 regulation were then studied in DU145 cells. Cultures were treated with DHT (5-10 nM) for 30 hours and probed for CXCR4 protein expression via Western blot and immunocytochemistry. Consistent with their AR-negative phenotype, treatment with androgens did not impact on CXCR4 protein expression in 2D cultures of DU145 cells. Treatment of cultures with 5-10 nM DHT had no impact on CXCR4 protein expression, when compared to controls via Western blot analysis (Fig. 4.22 A). Using an anti-CXCR4 antibody, immunocytochemistry studies demonstrated that the distribution of CXCR4 (shown in green) also remained consistent between controls (Fig. 4.22 B) and DHT-treated (10 nM) cultures (Fig. 4.22 C).

The androgen-insensitive DU145 cells retained a consistently AR-negative phenotype under 3D culture conditions, in contrast to PC3 cells. Accordingly, no impact on CXCR4 protein expression was observed after treatment with androgens in 3D culture. The expression of CXCR4 protein was consistent between control and DHT-treated (5-10 nM) cultures when analysed via Western blot (4.22 D). Immunocytochemistry studies using an anti-CXCR4 antibody demonstrated that the distribution and expression pattern of CXCR4 protein remained unchanged between controls (Fig. 4.22 E) and DHT-treated (10 nM) cultures (Fig. 4.22 F).
Figure 4.22 CXCR4 protein expression in DU145 cells was unaffected by androgen treatment in 2D or 3D culture.

DU145 cells were cultured in 2D (A-D) or 3D conditions (E-F) for 10 days prior to incubation in SFM alone, or containing the indicated concentration of DHT (5-10 nM) for 30 hours. Samples were collected and analysed for CXCR4 protein expression via Western blot (2D shown in A, 3D shown in D). Density was calculated in relation to the β-actin loading control. Using an anti-CXCR4 antibody, immunocytochemistry was performed on DU145 cultures. In 2D, the expression and distribution of CXCR4 protein (shown in green) was consistent between controls (B-B’) and cultures treated with DHT (10 nM) (C-C’). Immunocytochemistry studies in 3D also demonstrated that, in comparison with controls (E-E’), the expression of CXCR4 protein was not affected by DHT (10 nM) (F-F’). DRAQ5™ nuclear labelling is shown in red and CellMask™ blue in blue. Scale bar = 60µm. Results shown are representative of three separate experiments, with densitometry performed on representative data.
In summary, this research chapter comprised studies of CXCR4 regulation in 2D and 3D cultures of PCa cells, in relation to androgens and the AR. Studies of CXCR4 protein expression and functionality demonstrated a variation in receptor functionality between PCa cell lines. Interestingly, CXCR4 protein expression in PC3 cells was substantially enhanced in 3D culture, whilst expression of CXCR4 remained consistent between 2D and 3D cultures of LNCaP and DU145 cells. In addition, the functional response of CXCR4 to ligand stimulation was found to vary between androgen-sensitive and androgen-insensitive cells. Following SDF-1α stimulation, a strong intracellular signalling and cell migration response was observed in DU145 and PC3 cells. In comparison, the functional responses of CXCR4 in LNCaP cells were limited, and no cell migration response was observed. Furthermore, an AR-dependent regulation of CXCR4 protein expression by androgens detected in both LNCaP and PC3 cells. This regulation in PC3 cells required activity through the Src/MAPK signalling pathway, a finding that was not in common with LNCaP cells. DU145 cells displayed consistent protein expression in the presence and absence of DHT; an observation which was consistent with their AR-negative phenotype. Taken together, these results showed that CXCR4 protein expression was influenced by androgens in an AR-dependent manner in both LNCaP and PC3 cells, a regulation mechanism that was missing in the AR-negative DU145 cell line. Furthermore, the up-regulation of CXCR4 protein in response to androgens required activity through the distinct Src/MAPK signalling pathway in PC3 cells, a finding that was in contrast to LNCaP cells.
4.3 Discussion

4.3.1 SDF-1α treatment did not result in CXCR4 down-regulation

Down-regulation of CXCR4 in response to SDF-1α was not observed via Western blot in LNCaP (Fig. 4.1 B-C), PC3 (Fig. 4.9 B-C) and DU145 cells (Fig. 4.17 B-C), most likely because images were obtained after 24 hours of ligand treatment. CXCR4 down-regulation has previously been shown to occur after 30-150 minutes after treatment with SDF-1α, due to inefficient recycling as a consequence of ligand-induced receptor degradation (Tarasova et al. 1998). The time-course of SDF-1α performed in a previous study showed that SDF-1α resulted in up-regulation of CXCR4 mRNA after 6 hours of treatment (Kukreja et al. 2005), however a time-course of 6, 12, 24 hours in our hands showed the protein expression of CXCR4 to be maintained at all of these timepoints (data not shown). The 24 hour time-point was shown for all cell lines to provide a representative image to demonstrate that CXCR4 expression was maintained. However the likely reason for this difference compared to the study by Kukreja and colleagues (2005) was that the dose of SDF-1α used here was too low to trigger enhanced CXCR4 expression in culture. In the current study, 50 ng/ml SDF-1α was used, versus 100 ng/ml in the study by Kukreja and colleagues (2005).

4.3.2 Differences in CXCR4 Function between PCa Cell Lines

Previous studies have shown that androgen-sensitive LNCaP cells display lower adhesion to ECM proteins than the DU145 and PC3 cell lines, combined with a low invasive phenotype and limited tumour-forming ability in mice (Bonaccorsis 2000; Witkowski 1993). Therefore, it was expected that LNCaP cells would display lower levels of CXCR4 protein expression than the more invasive androgen-insensitive PCa cell lines (Bonaccorsi 2000). LNCaP cells cultured in 2D displayed a clear expression of CXCR4 protein; however, whilst CXCR4 was observed to respond to SDF-1α and efficiently internalise into the cytoplasm, the downstream effects of receptor stimulation were minimal. Stimulation of LNCaP cells with SDF-1α did not elicit a signalling response in the PI3K/Akt or MAPK pathways, after up to 60 minutes of stimulation. The lack of PI3K/Akt pathway activation was consistent with previously published data (Wang et al. 2005a).
However, the absence of an ERK phosphorylation reported here was in contrast to results by Wang and colleagues (2005a). Although Wang (2005a) reported the phosphorylation of ERK 1/2 after an extended 1-4 hour stimulation with SDF-1α, typically other PCa cell lines display a more rapid ERK 1/2 phosphorylation response, within 5-30 minutes of ligand stimulation (Huang et al. 2007; Kukreja et al. 2005; Sun 2003). The maximum time period used in the current study was 60 minutes, which was a likely reason the ERK 1/2 phosphorylation response was not detected in our experiments. The protocol used in the current study was optimised to observe signalling responses in DU145 and PC3 cells, and consistent time periods were employed between the cell lines tested to enable comparisons. Under the chosen conditions, the downstream signalling in the LNCaP cell line occurred to a lesser extent when compared to PC3 and DU145 cells.

Studies of Akt phosphorylation in LNCaP cells in response to SDF-1α revealed two bands, as opposed to a single band on a Western blot (Fig. 4.2 A). This result was observed in all three replicates of the experiment (n=3). The major upper band was used for quantification via densitometry, because this band specifically corresponded to the size of the band for phospho-Akt which was evident on re-probing.

The lack of cell migration response towards SDF-1α in the LNCaP cell line was in contrast to the results reported by Frigo and colleagues (2009). Multiple attempts to re-create the methodologies detailed by Frigo (2009) were unsuccessful in generating a cell migration response to SDF-1α in this cell line. Considering the low invasive phenotype and adhesive properties of LNCaP as reported in previous studies, the lack of cell migration in LNCaP observed in the current study was consistent with the less aggressive phenotype of the cell line (Witkowski 1993). When considered in combination with the lack of PI3K/Akt and MAPK pathway signalling after SDF-1α treatment, those results revealed that ligand-stimulated CXCR4 may be operating at a lower level of activity in LNCaP cells in comparison to DU145 and PC3 cells under the conditions tested.

Accordingly, perhaps the low-invasive phenotype of LNCaP cells in the aforementioned in vivo studies may be linked to the limited function of ligand-stimulated CXCR4 in this cell line (Bonaccorsi 2000; Witkowski 1993). Rather than observing functional responses to be directly associated with CXCR4 protein
expression levels between the cell lines studied, the limited functionality of CXCR4 in LNCaP cells may be a consequence of altered integrin expression and regulation in this cell line. Previous studies have demonstrated that the interactions of specific integrins with the surrounding ECM are essential for CXCR4-induced cell adhesion activities of DU145 and LNCaP cells (Engl et al. 2006). In those cell lines, CXCR4-induced cell adhesion to ECM proteins was mediated through up-regulation and activation of α5 and β3 integrins in response to SDF-1α (Engl et al. 2006). An earlier comparative study of integrin subunit expression and function between the DU145, PC3 and LNCaP cell lines demonstrated a direct relationship between the adherence of LNCaP cells with their altered integrin expression profile (Witkowski 1993). Specifically, the comparatively lower expression of the α3 integrin subunit in LNCaP cells significantly impacted on cell adhesion to fibronectin, laminin and vitronectin (Witkowski 1993). This linked to a lower tumorigenicity of the LNCaP cells in vivo (Witkowski 1993). Considering the reliance of tumour cell migration and invasion on successful cell adhesion and interaction with the ECM, the inability of LNCaPs to undergo CXCR4-mediated cell migration to SDF-1α in the current study may have been a consequence of their limited integrin expression profile (Clarke et al. 2009; Witkowski 1993).

4.3.3 CXCR4 Function in DU145 and PC3 Cells

For both DU145 and PC3 cell lines, SDF-1α-induced signalling was followed by a strong CXCR4-mediated cell migration response. Activation of the PI3K/Akt pathway was observed in both DU145 and PC3 cell lines after SDF-1α stimulation, whilst the ERK/MAPK pathway was activated only in DU145 cells. Interestingly, when those signalling responses were tested for their reliance on CXCR4 activity, it was found that Akt phosphorylation in both cell lines was not dependent solely on CXCR4. No inhibition of the SDF-1α-induced Akt phosphorylation response was observed in both PC3 and DU145 cells treated with AMD3100. That indicated that the response was not dependent on CXCR4 activity, and that SDF-1α stimulation may have activated alternative pathways to elicit Akt phosphorylation. For example, it is now known that a second SDF-1α binding receptor, CXCR7, can activate Akt in response to SDF-1α in the androgen-insensitive LNCaP C4-2B cell line (Wang et al. 2008a). Considering
that CXCR7 is an alternative SDF-1α binding receptor that was found to be expressed on LNCaP cells (Wang et al. 2008a), it was possible that CXCR7 may have contributed to the Akt pathway activation observed.

In comparison, the cell migration response of both DU145 and PC3 cells was found to be mediated by CXCR4. Using AMD3100, the cell migration response to SDF-1α was prevented in both DU145 and PC3 cell lines.

4.3.4 Androgen Regulation of CXCR4 in PCa Cells

AR-positive PC3 cells in 3D culture displayed a positive regulation of CXCR4 in response to androgen treatment, similar to that observed in LNCaP cells. Both AR protein expression and activity were required for this effect in PC3 cells, as it was specific to 3D cultures and was inhibited by incubation with bicalutamide. Bicalutamide inhibits AR activation by binding directly to the AR ligand-binding domain, preventing association with androgen ligands and downstream co-activators required for transcriptional activity (Osguthorpe et al. 2011). Therefore, it was likely that the effectiveness of bicalutamide at inhibiting androgen-induced CXCR4 protein up-regulation was due to its action in preventing the binding of AR to DHT. This, in turn, prevented receptor activation and subsequent signalling pathway activation.

Non-genomic pathways downstream of AR activation include kinase cascades, calcium signalling and cytoskeletal re-arrangements, generating changes to cell phenotype (Hatzoglou et al. 2005; Kampa et al. 2002; Papakonstanti et al. 2003). These rapid signalling pathways have been associated with the emergence of CRPC (Unni 2004). Unni and colleagues (2004) demonstrated that an androgen-insensitive subline of LNCaP cells (LNCaP-HP) displayed enhanced Src/MAPK signalling, which was linked with an increased resistance to apoptosis (Unni 2004). In the current study, the lack of PSA production observed in 3D cultures of PC3 cells (Chapter 3) indicated that AR activation in PC3 does not result in enhanced PSA production. Potential factors that may influence the transcriptional activation of the PSA gene include gene methylation (Park 2010a), altered expression or activity of transcriptional cofactors (Itkonen et al. 2012), or the possible existence of single-nucleotide polymorphisms in the PSA which may impact on functional transcription factor binding (Shastry 2009). In the current
chapter, it was established that Src/MAPK pathway activity was required for AR-mediated regulation of CXCR4. When these findings were considered together, this indicated that androgenic regulation of CXCR4 was mediated through the Src/MAPK pathway in PC3 cells. In contrast, the observations in LNCaP cells demonstrated Src/MAPK pathway was unlikely to play a role in AR-mediated regulation of CXCR4 in this cell line. Collectively, this indicated that CXCR4 regulation by androgens was mediated through different signalling pathways between PC3 and LNCaP cells.

Combined with the lack of PSA production observed in AR-positive 3D cultures of PC3 cells, perhaps non-genomic signalling downstream of AR activation contributed to the regulation of CXCR4 in this cell line. However, bicalutamide treatment was observed to ameliorate the up-regulation of CXCR4 in response to DHT. Previously, it has been shown that non-steroidal anti-androgens such as bicalutamide can block the genomic functions of AR and have no impact on rapid, non-genomic signalling (Papadopoulou et al. 2008; Papakonstanti et al. 2003). Therefore, the fact that bicalutamide inhibited the response indicated that genomic functions downstream of AR activation may play a role in regulation of CXCR4 in 3D cultures of PC3. Further investigations are required to determine the impact of AR activation on other AR-target genes in PC3 cells, in order to permit a comparison with the downstream AR activation processes with that of LNCaP.

4.3.5 Measuring the Functional Consequences of CXCR4 Modulation in 3D Cultures

After observing an enhanced expression of CXCR4 protein by androgens in 3D cultures of PC3 cells, the resulting functional consequences were studied in cell migration assays. In order to utilise 3D cultures in a Transwell® migration assay format, a cell extraction step was required to dissociate 3D spheroids and remove cells from within the Matrigel matrix. The cell extraction procedure available for this purpose was the use of BD™ Cell Recovery Solution (CRS; BD Biosciences). In this protocol, cells were incubated at 4°C to keep the Matrigel in liquid form, in addition to multiple wash steps and centrifugations. Efforts to perform cell migration assays with these extracted cell populations yielded no detectable cell migration response towards SDF-1α (data not shown).
The most likely reason for this lack of response was the cell extraction process itself. Considering that PC3 cells are capable of a strong cell migration response to SDF-1α in 2D culture, it was possible that the growth of PC3 cells in 3D culture may result in a decreased migration response. However, the health of cells appeared to be adversely affected upon microscopic examination subsequent to processing via the CRS cell extraction protocol. The complete absence of cell migration activity in these extracted cells suggested that the trauma exerted by the cell extraction process itself contributed to the lack of cell migration response. Perhaps cell migration may be assessed successfully in a format that does not require the extraction of cells from 3D culture in the Matrigel matrix, in order to permit measurement of the effect of androgens on CXCR4 functionality.

In summary, the results in this chapter identified differences in the regulation of CXCR4 protein expression between PCa cell lines in 2D and 3D culture. The functional response of CXCR4 was variable between PCa cell lines, and the capacity to undergo CXCR4-mediated cell migration did not directly correlate with CXCR4 protein expression. The culturing of PC3 cells in 3D resulted in enhanced CXCR4 protein expression, whereas in DU145 and LNCaP cells the expression in 2D and 3D culture remained consistent. Furthermore, these results demonstrated that AR-mediated regulation of CXCR4 by androgens can occur in AR-positive PCa cells, irrespective of androgen-sensitive or androgen-insensitive status. CXCR4 protein expression was influenced by androgens in an AR-dependent manner in both AR-positive LNCaP and PC3 cells, a regulation mechanism that was missing in the AR-negative DU145 cell line. Furthermore, the up-regulation of CXCR4 protein in response to androgens required activity of distinct signalling pathways in PC3 when compared to LNCaP cells. As our data indicated that AR in PC3 cells may display limited transcriptional activity, it was likely that regulation of CXCR4 in PC3 cells occurred through different pathways downstream of AR activation, which could be mediated through either the genomic or non-genomic effects of androgens. Therefore, the implications of these findings are that AR antagonists targeting both upstream receptor activities and downstream transcriptional activation may be more effective at inhibiting the role of AR in the growth and progression of PCa.
5 CHAPTER FIVE: CONSTRUCTION OF AN INDUCIBLE AR EXPRESSION SYSTEM
5.1 Introduction

Previous studies have shown that AR may regulate CXCR4 in androgen-insensitive cell lines in systems of over-expression \textit{in vitro} (Akashi \textit{et al.} 2006). To date, however, limited data is available in literature to describe this regulation. Transfection of DU145 cells with an AR construct resulted in a down-regulation of CXCR4 mRNA expression and functional migration in response to SDF-1\(\alpha\) (Akashi \textit{et al.} 2006). Transfection of PC3 cells with AR also resulted in decreased adhesion and invasive capacity, which was linked to altered integrin expression profiles (Bonaccorsi \textit{et al.} 2004). These findings suggested that androgenic regulation of chemokine receptors inhibited invasive phenotype in PC3 cells.

In contrast, Nagakawa and colleagues (Nagakawa 2004; Nagakawa 2002) reported that the invasive ability of DU145 cells was enhanced upon transfection with AR. DU145-AR cells have also been found to display altered expression of integrins and pro-angiogenic cytokines (Nagakawa 2004; Nagakawa 2002). Specifically, the expression of \(\alpha6\) and \(\beta4\) integrin subunits were down-regulated, while expression of \(\alpha2\) and \(\alpha5\) subunits were up-regulated (Nagakawa 2004). The altered expressions of these integrins were reflected in lower adhesion of these cells to fibronectin, laminin-1 and laminin-5, with enhanced adhesion to collagens (Nagakawa 2004). An increased ability of the DU145-AR cells to invade through Matrigel was also observed, which was further enhanced through treatment with androgens (Akashi \textit{et al.} 2006).

Considered together, these reports demonstrate that AR can impact on invasive capacity in PCa cell lines; however, the contradictory results indicate that androgenic regulation of invasive behaviour is largely undetermined. It is unclear whether the activity of AR acts to encourage or suppress the invasive activities in androgen-insensitive PCa cells. Regulation of the chemokine receptor network, in particular, was of interest in the androgen-insensitive stage of disease, to more clearly elucidate the mechanisms governing the regulation of cancer cell growth and dissemination. The aim of the current chapter was to generate androgen-insensitive PCa cells stably expressing AR, to use in studies of CXCR4 regulation.
5.2 Results

5.2.1 Generating the Tetracycline-Inducible AR Expression Construct

The PSG5-AR vector (generously provided by Dr Chang, University of Rochester, USA) was a transient expression vector that did not contain antibiotic selection markers, and was therefore suitable for use in transient transfections. The PSG5-AR vector was analysed by DNA sequencing analysis (Appendix C), and the sequence of the AR insert in PSG5-AR provided 100% sequence alignment with wild-type human AR transcript mRNA (accession number NM000044.3; (Nucleotide: National Centre for Biotechnology Information 2009)) as verified using the Basic Local Alignment Search Tool (BLAST: National Centre for Biotechnology Information 2009). However, for use in transfections, a stable expression vector was desired, in order to obtain the most consistent, reproducible results. A tetracycline-inducible expression system was chosen for stable expression of AR in PCa cell lines, using the regulatory vector pcDNA™6/TR and the inducible expression vector pTREX™-DEST30 (Fig. 5.1 A). This system allowed for AR expression to be induced upon application of the antibiotic, tetracycline (Fig. 5.1 B-C).
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Figure 5.1 The T-REX™ tetracycline-inducible expression system.
The pTREX™-DEST30 vector contained a Tet-Operator element (Tet 0₂), and pcDNA™6/TR contained the Tet-Repressor (Tet-R) element (A). When these vectors were co-transfected into mammalian cells, the interaction between Tet0₂ and Tet-R enabled the expression of the gene of interest to be regulated by the application of tetracycline. In the absence of tetracycline, Tet-R was bound to the Tet0₂ element upstream of the CMV promoter (pCMV), inhibiting transcription of the gene of interest (B). In the presence of tetracycline, the binding of the Tet-R to Tet0₂ was prevented, permitting the activation of gene expression (C). Figure (A) was adapted from (T-REX™ system user manual: Life Technologies 2002) and (pcDNA™6/TR user guide: Life Technologies 2009). Figure (B) was adapted from Jones et al (2005).
5.2.1.1 Confirming PSG5-AR Vector Identity and Function

The PSG5-AR vector was used to transiently transfect both DU145 and PC3 cell lines to confirm the expression of AR. Whilst DU145 and PC3 cells transfected with empty PSG5-vector did not express detectable AR protein, those transfected with PSG5-AR displayed AR protein expression, as shown via Western blot (Fig. 5.2 A). Use of an anti-AR antibody in immunocytochemistry studies demonstrated that a nuclear translocation of AR protein was observed in response to DHT (100 nM) treatment (Fig. 5.2 B).

![Western Blot and Immunocytochemistry Images]

Figure 5.2 Transfection of cells with PSG5-AR produced androgen-responsive AR protein. PC3 cells were transfected with PSG5-empty or PSG5-AR vector and analysed for AR protein expression via western blot (A). The localisation of AR protein was investigated in PC3 cells using an anti-AR antibody via immunocytochemistry. In controls (B), AR localisation (shown in green) was diffuse throughout the cytoplasm and nucleus. Stimulation with DHT (100 nM, 24 hours) resulted in nuclear translocation of AR. DRAQ5™ staining of nuclei is shown in red. Scale bar = 10 µm. The splicing of the Western blot results in (A) was performed in order to condense the results, removing extra samples which resided between these conditions on the same Western blot. Results shown are representative of three separate experiments.

5.2.1.2 Constructing the Tetracycline-Inducible AR Expression Vector

The cloning of the AR gene open reading frame into the tetracycline inducible expression vector pTREX™-DEST30 (Life Technologies) was performed using Gateway® cloning technology (Fig. 5.3). Firstly, this involved cloning of the AR gene into an entry vector, from which the gene can be directionally cloned into a destination vector, using a propriety Clonase™ LR recombination reaction (Life Technologies).
In order to facilitate cloning of the AR sequence into the tetracycline-inducible pTREX™-DEST30 vector, the AR gene was cloned into an intermediate entry vector (pENTR™/D-TOPO®). Initially, PCR was used to amplify the AR sequence from PSG5-AR via using AR-specific primers (A). The primers were designed to incorporate additional restriction sites onto the 5' (NotI) and 3' (AscI) ends of the amplified copies of the AR gene. Subsequent to digestion of the plasmid DNA with NotI and AscI restriction enzymes (B), the amplified DNA was then compatible for direct ligation into the pENTR™/D-TOPO® plasmid (C). Once AR was successfully cloned into this entry vector, a Clonase™ LR recombination reaction was used to transfer the AR gene into the tetracycline-inducible AR expression vector (pTREX™-DEST30-AR) (D). Figure was adapted from (T-REX™ system user manual: Life Technologies 2002).
5.2.1.2.1 Primary Cloning Approach: PCR

Originally, attempts at transferring the AR gene from PSG5-AR into the entry vector (pENTR™/D-TOPO®) were based on the use of PCR to amplify the gene prior to ligation into the vector (Fig. 5.3). The PCR primers were designed to incorporate additional nucleotides onto the 5’ and 3’ ends of the AR amplicon, creating restriction enzyme sites for NotI and AscI, respectively. These additional sites provide compatible ends for ligation into the digested pENTR™/D-TOPO® vector, to facilitate directional cloning. However, despite the use of a high-fidelity DNA polymerase, PCR amplification of the AR gene was problematic. Clones generated using this method were found to contain numerous base pair errors. The potential reasons for this include the extensive length of the AR gene (and therefore the length of PCR amplicons; 2.7 kb), in addition to the GC-rich nature and numerous repeat regions within the sequence (Appendix C). Consequently, an alternative method was employed to clone AR into the pENTR™/D-TOPO® vector, which avoided the use of PCR.

5.2.1.2.2 Alternative Cloning Approach: Restriction Enzymes and Linkers

A restriction digestion approach was used to clone AR into pENTR™/D-TOPO®. Excision of the AR gene from PSG5-AR was performed using the Bgl II and BamHI restriction enzyme sites, which reside at the 5’ and 3’ end of the AR gene, respectively. However, due to a lack of Bgl II and BamHI sites present in the pENTR™/D-TOPO® vector, direct ligation of the excised AR gene into pENTR™/D-TOPO® was not possible. The restriction enzyme sites for Bgl II and BamHI were engineered into the pENTR™/D-TOPO® vector prior to ligation, to facilitate directional cloning of AR into the vector.

5.2.1.2.3 Addition of Compatible Restriction Sites into pENTR™/D-TOPO®

An oligonucleotide linker approach was utilised to engineer additional restriction sites into the pENTR™/D-TOPO® vector (Fig. 5.4). The linkers were short, 25 base pair oligonucleotides that were designed and commercially synthesised to contain the Bgl II and BamHI restriction sites (Table 2.5). The incorporation of this linker into pENTR™/D-TOPO® was facilitated by specific base pair overhangs in the linker sequence, compatible for ligation into the NotI and AscI restriction sites in the digested pENTR™/D-TOPO® vector (Fig. 5.4).
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Figure 5.4 Addition of Bgl II and BamHI restriction enzyme sites into the pENTR™/D-TOPO® vector using oligonucleotide linkers.

In order to facilitate directional cloning of the AR gene into the pENTR™/D-TOPO® vector, additional restriction enzyme sites for Bgl II and BamHI were engineered into the vector sequence. These sites were engineered into the pENTR™/D-TOPO® vector using linkers, short sequences of DNA which were designed to be 25 bases in length. When annealed together as a double-stranded sequence, these linkers contained restriction enzyme sites for Bgl II and BamHI. The ligation of the linkers into the pENTR™/D-TOPO® vector was mediated by specific base-pair overhangs, that were designed for compatible ligation with the NotI and AscI restriction sites already present in the vector. Once pENTR™/D-TOPO® was digested with NotI and AscI (A), the reaction products were incubated in a ligation reaction with the double-stranded linker (B). Subsequent to incorporation of the linker sequence into pENTR™/D-TOPO®, the vector contained additional Bgl II and BamHI restriction sites, which would then facilitate cloning of the AR gene into the vector. Figure adapted from (Gateway® pENTR™ vector manual: Life Technologies 2012).
The oligonucleotide linkers were annealed together to create a double-stranded DNA linker (DS-linker). Subsequent to digestion of the pENTR™/D-TOPO® vector with NotI and AscI, the DS-linker was incubated in a ligation reaction with the digested vector. To confirm that the linker had been incorporated into the pENTR™/D-TOPO® vector, the ligation product was propagated in E.coli, purified and subject to restriction enzyme analysis. Consistent with incorporation of the DS-linker sequence into the pENTR™/D-TOPO® vector, Bgl II (Fig. 5.5 A) and BamHI (Fig. 5.5 B) digestions produced the expected size of DNA fragments when visualised on an agarose gel.

**Figure 5.5 Confirmation of linker incorporation into the pENTR™/D-TOPO®.**

The pENTR™/D-TOPO® vector sequence did not feature restriction sites for Bgl II or BamHI, however the ligation of linkers into pENTR™/D-TOPO® provided these additional restriction sites. To confirm successful incorporation of linkers into the pENTR™/D-TOPO® vector, plasmid preparations of the pENTR™/D-TOPO®-linker (pENTR™-linker) ligation products were incubated with Bgl II or BamHI for 2 hours at 37 °C. The reaction products were analysed on a 1.5% (w/v) agarose gel and visualised with ethidium bromide. Whilst the undigested pENTR™-linker is shown in lane 4, the linearisation of pENTR™-linker clones by Bgl II (A) and BamHI (B) evident in lane 3 demonstrated the presence of the linker sequence. Included as a positive control, the PSG5-AR vector contained restriction sites for both the Bgl II and BamHI enzymes, demonstrating that the enzymes were functional under these conditions.
5.2.1.2.3.1 Generating the pENTR™-AR Entry Vector

Using the Bgl II and BamHI restriction enzyme sites flanking the AR gene, the AR sequence was excised from the PSG5-AR vector and ligated into the Bgl II and BamHI sites now present in the pENTR™/D-TOPO® plasmid (Fig. 5.4). This method produced pENTR™/D-TOPO® clones, which were then analysed for the presence of the AR insert via restriction enzyme analysis. Bgl II, BamHI and HindIII were used to distinguish between successful and unsuccessful ligation clones (Fig. 5.6); whilst the pENTR™/D-TOPO® vector itself does not contain restriction sites for these enzymes, they are present in the AR insert itself (Gateway® pENTR™ vector manual: Life Technologies 2012; Litvinov et al. 2004).

![Figure 5.6 Restriction enzyme analysis of pENTR™-AR ligation clones.](image)

Plasmid preparations of pENTR™-AR ligation clones 1 and 2, or the positive control PSG5-AR, were incubated with Bgl II, BamHI and/or HindIII restriction enzymes for 2 hours at 37°C. Reaction products were analysed on a 1.5% (w/v) agarose gel and visualised with ethidium bromide. These enzymes were chosen because they are present in the AR insert but not in the pENTR™/D-TOPO® vector itself, providing the ability to distinguish between ligation clones that contain the insert and those that do not. The undigested vectors were analysed in parallel with the digested vector samples. Due to the consistent size of clone 1 between undigested (lane 1) and digested vector samples (lanes 2 and 3), it was evident that clone 1 did not contain these restriction sites. In contrast, clone 2 (lane 4) was digested successfully by Bgl II, BamHI and/or HindIII (lanes 5 and 6), demonstrating the presence of restriction sites for these enzymes. The approximate bp size of the reaction products, as determined with reference to the ladder, was consistent with the presence of the AR insert in pENTR™/D-TOPO® vector in clone 2 (lane 5 = 3,000 bp and 2,580 bp; lane 6 = 1,580 bp and 4,000bp. Included as a positive control, the PSG5-AR vector (lane 7) was successfully digested by the enzymes (lanes 8 and 9), which demonstrated that these restriction enzymes were functional under the conditions tested.
As shown in Fig. 5.6, ligation clone 1 remained undigested in the presence of these enzymes; however clone 2 was successfully digested by \textit{Bgl II}, \textit{BamHI} and \textit{HindIII}. The DNA fragment sizes produced by clone 2 were consistent with the presence of the AR insert in the pENTR$^{TM}$/D-TOPO® vector: The pENTR$^{TM}$/D-TOPO® vector itself was 2,580 bp in size, and incorporation of the 2,763 bp AR insert would make it 5,343 bp in total size. Due to the positioning of the restriction enzyme sites within the vector, double digestion with \textit{Bgl II} and \textit{BamHI} would produce two fragments of approximately 3,000 bp and 2,580 bp, and digestion with both \textit{Bgl II} and \textit{HindIII} would produce two DNA fragments of approximately 1,580 bp and 4,000 bp.

The restriction profile of clone 2 was consistent with that expected of the successfully ligated pENTR$^{TM}$-AR construct. Subsequent analysis via DNA sequencing confirmed the presence of the AR insert in the clone 2 (Appendix D). This clone was then used in a Clonase$^{TM}$ LR recombination reaction to insert the AR gene into the final pTREX$^{TM}$-DEST30 expression vector.

\textit{5.2.1.2.3.1 Generating the pTREX$^{TM}$-DEST30-AR Expression Vector}

The pENTR$^{TM}$-AR clone 2 clone was then used in the subsequent Clonase$^{TM}$ LR recombination reaction. The \textit{AttL} sites adjacent to the AR insert within pENTR$^{TM}$/D-TOPO® undergo a site specific recombination reaction with the \textit{AttR} sites in the pTREX$^{TM}$-DEST30 vector (T-REX$^{TM}$ system user manual: \textit{Life Technologies} 2002). Accordingly, this reaction enables directional cloning of the AR insert into the pTREX$^{TM}$-DEST30 expression vector (Fig. 5.7).
Figure 5.7 Construction of the pTREX™-DEST30-AR expression vector.

Prior to cloning the AR sequence into the tetracycline-inducible expression vector pTREX™-DEST30 using Gateway® technology, AR was cloned into an intermediate entry vector (pENTR™/D-TOPO®). The Bgl II and BamHI restriction enzyme sites, which flanked the AR gene, were selected to facilitate the cloning of the AR gene from PSG5-AR into the pENTR™/D-TOPO® entry vector. After compatible restriction sites were engineered into the pENTR™/D-TOPO® vector using linkers, the pENTR™/D-TOPO®-linker vector was digested with Bgl II and BamHI (A). Using these same restriction enzymes, the AR sequence was excised from PSG5-AR (B) and ligated into the digested pENTR™/D-TOPO® vector (C). The resulting pENTR™/D-TOPO®-AR vector was then incubated in a Clonase™ LR recombination reaction to mediate the transfer of AR into the final, tetracycline-inducible pTREX™-DEST30 expression vector. This enzyme was used to facilitate a site-specific recombination reaction between AttL and AttR sites, which were present in both the entry (pENTR™/D-TOPO®) and destination vectors (pTREX™-DEST30) (D). Figure adapted from (T-REX™ system user manual: Life Technologies 2002).
After incubation of pENTR™-AR clone 2 in the Clonase™ LR recombination reaction with pTREX™-DEST30, the reaction products were propagated in *E.coli*. Following plasmid purification, the presence of the AR insert was confirmed via restriction enzyme analysis. The AR insert provided an additional *HindIII* restriction site, ensuring the pTREX™-DEST30-AR vector would produce three DNA fragments (1 x 1,350 bp, 1x 1,672 bp, 1x 6,258 bp) upon digestion with *HindIII*. In comparison, the unmodified pTREX™-DEST30-vector (herein referred to as pTREX™-DEST30-empty) would generate two DNA fragments (1 x 1,672 bp, 1x 5,872 bp) after digestion with *HindIII*. The ligation clone 1 was observed to produce three DNA fragments at the expected size after digestion with *HindIII* (Lane 4, Fig. 5.8), consistent with the presence of the AR sequence. Subsequently, DNA sequencing analysis was used to confirm the identity of the insert as the AR sequence, to verify that the pTREX™-DEST30-AR vector had been successfully constructed.

![Figure 5.8](image)

**Figure 5.8** Restriction enzyme analysis of the pTREX™-DEST30-AR ligation clone 3.

The ligation product (clone 3) was propagated in *E.coli* and purified, prior to incubation with *HindIII* restriction enzyme for 2 hours at 37°C. The reaction product was analysed via agarose gel electrophoresis (1.5% w/v) and visualised with ethidium bromide. Included as a comparison, the pTREX™-DEST30-empty vector contained two *HindIII* restriction enzyme sites, and produced DNA fragments of 1672 bp and 5,872 bp after digestion with this enzyme (Lane 2). The AR insert contained an additional *HindIII* restriction enzyme site, and ligation clones that contained the AR insert would produce three fragments of 1,350 bp, 1,672 bp and 6,258 bp after digestion with *HindIII* (Lane 4). Three DNA fragments were observed after the digestion of clone 3 with *HindIII*, which indicated that ligation clone 3 had successfully incorporated the AR sequence.
5.2.1.3 Validation of Expression Vector Function

Prior to the use of the pTREX\textsuperscript{TM}-DEST30-AR vector in subsequent investigations, it was necessary to confirm that this vector produces intact and functional AR protein when expressed in PCa cells. Transient co-transfections of pTREX\textsuperscript{TM}-DEST30-AR, pTREX\textsuperscript{TM}-DEST30-empty and/or pcDNA\textsuperscript{TM}6/TR were performed in DU145 and PC3 cell lines, and cultures were incubated in the presence of tetracycline (1 mg/ml; 24 hours) prior to analysis of AR protein expression. Western blot analysis of whole cell lysates demonstrated that, whilst cells containing the pTREX\textsuperscript{TM}-DEST30-empty vector did not express AR protein, cultures transfected with pTREX\textsuperscript{TM}-DEST30-AR showed a clear induction of AR protein in the presence of tetracycline (Fig. 5.9 A). When analysed via immunocytochemistry using an anti-AR antibody, AR was observed as diffuse staining throughout the cytoplasm and nucleus in controls (Fig. 5.9 B). Upon treatment with DHT (10 nM; 24 hours), a nuclear translocation response was evident (Fig. 5.9 B). Production of the AR target-gene, PSA, was then assessed by RT-PCR. RNA was extracted from cultures, transcribed into cDNA and subject to PCR using gene specific primers for AR and PSA. In the presence of tetracycline, AR mRNA expression was induced along with PSA mRNA (Fig. 5.9 C). This demonstrated that transfection of DU145 cells with pTREX\textsuperscript{TM}-DEST30-AR resulted in the expression of functional AR mRNA and protein.
Figure 5.9 Transient transfection with pTREX™-DEST30-AR produced ligand-responsive AR expression.

DU145 cells were co-transfected with either pTREX™-DEST30-AR or pTREX™-DEST30-empty and pcDNA™6/TR, and incubated for 16 hours for recovery prior to stimulation with 1 mg/ml tetracycline (+/- 10 nM DHT) for 24 hours. Cells were analysed for AR expression via western blot (A) or fixed and processed for AR expression via immunocytochemistry (B). An anti-AR monoclonal antibody was used to label AR (shown in green) and nuclei were stained with DRAQ5™ (shown in red). RNA extractions and RT-PCR were performed on transfected DU145 cells, using gene-specific primers for AR and PSA (C). PCR products were analysed using agarose gel electrophoresis and stained with ethidium bromide. LNCaP samples were included as a positive control to demonstrate the primers for AR and PSA were functional, producing reaction products as expected. Scale bar = 50 µm.
5.2.2 Optimisation of Cell Transfection Protocol

Inducible protein expression was obtained by co-transfection of the pTREX™-DEST30-AR vector into cells, in combination with pcDNA™6/TR. In order to select for stable transfectants, cells were incubated in the presence of selection antibiotics specific for the vector of interest; G418 for pTREX™-DEST30-AR, and Blasticidin for the pcDNA™6/TR vector. Optimisation of selection antibiotic dosage was performed in both the PC3 and DU145 cell lines, to determine the optimal concentrations of antibiotic required to cause toxicity in non-transfected cells and therefore allow selection of transfected cells in culture (Fig. 1.2).

The optimal concentrations of G418 required to kill non-transfected cells was found to be 500 µg/ml (PC3) and 800 µg/ml (DU145). The concentrations for selection using Blasticidin were found to be optimal at 7 µg/ml (PC3) and 6 µg/ml (DU145). Post-transfection, these concentrations of G418 and/or Blasticidin were included in culture medium for a period of two weeks in order to select for transfected cells. Following initial selection, the dosage of selection antibiotic was slightly decreased to a maintenance concentration, and used consistently during future culturing of these cell populations in order to maintain selective pressure for transfectants in culture. G418 concentrations were reduced to 400 µg/ml (PC3) and 500 µg/ml (DU145), and Blasticidin to 6 µg/ml (PC3) and 5 µg/ml (DU145) for maintaining these cultures.

However, upon examination of cultures after completion of antibiotic selection, the consistency and maintenance of AR protein expression was a concern. Although some of the transfected cell populations initially expressed AR at a detectable level, the protein expression of AR within the population was highly heterogeneous, and was lost within 2-3 passages of the culture (Fig. 5.10). Despite re-optimisation of the transfection and selection process, this issue was encountered in repeated batches of transfectants. Subsequently, a transient transfection approach was selected for investigations using the pTREX™-DEST30-AR vector.
Chapter Five: Construction of an Inducible AR Expression System

5.2.2.1 Testing the Effect of Tetracycline on Cell Viability

Prior to further investigations in this tetracycline-inducible expression system, the natural tolerance of DU145 cells to tetracycline was assessed, to ensure that doses applied to transfected cells were not likely to compromise cell viability. Non-transfected DU145 cells were incubated with 0.5-5 µg/ml tetracycline for 24 hours, or with 1-10% (v/v) DMSO as a positive control. After 24 hours, the viability of cultures were assessed using an MTT assay, by the addition of MTT (10% v/v) solution directly into wells, re-solubilisation in DMSO, and reading absorbance at 570 nm. In the positive control wells, 5-10% (v/v) DMSO treatment resulted in decreased signal, reflecting an expected reduction in cell viability (Fig. 5.11). By comparison, the addition of tetracycline at concentrations between 0.5-5 µg/ml did not affect cell viability readings (Fig. 5.11). From these data, there was no evidence of tetracycline-induced toxicity up to a concentration of 5 µg/ml.
Figure 5.11  Tetracycline treatment did not produce toxicity in DU145 cells.

An MTT assay was used to assess the effect of tetracycline on cell viability. Cells were plated in complete medium and treated with tetracycline (0.5-5 µg/ml) or DMSO (5-10%; positive control) for 24 hours at 37°C. MTT solution was then added directly to wells (10% v/v), and incubated for 2 hours at 37°C, prior to solubilisation in DMSO and reading absorbance at 570 nm. Error bars denote S.E.M. Results shown are representative of three separate experiments.
5.2.2.2 Optimisation of Vector Ratios for Transient Co-Transfection

Tetracycline-inducible expression required the integration of two vectors into the same cell after co-transfection. The pTREX\textsuperscript{TM}-DEST30-AR vector provided AR expression, whilst co-transfection with the pcDNA\textsuperscript{TM}6/TR vector provided inducible gene expression in the presence of tetracycline. In the absence of pcDNA\textsuperscript{TM}6/TR, the pTREX\textsuperscript{TM}-DEST30-AR vector provided constitutive AR expression. A low basal expression of AR was required in the absence of tetracycline, and this was be achieved through higher expression of the regulatory tet-repressor, provided in the pcDNA\textsuperscript{TM}6/TR vector (pcDNA\textsuperscript{TM}6/TR user guide: Life Technologies 2009). Therefore, it was necessary to transfect a larger μg amount of pcDNA\textsuperscript{TM}6/TR DNA than the pTREX\textsuperscript{TM}-DEST30 expression vector (3-fold to 12-fold) during the co-transfection process.

For these assays, the control pTREX\textsuperscript{TM}-DEST30-LacZ plasmid was used to generate an easily detectable inducible gene product, β-Galactosidase. A ratio of 6:1 (μg pcDNA\textsuperscript{TM}6/TR: μg pTREX\textsuperscript{TM}-DEST30-LacZ) for transfection was recommended by the manufacturer (Life Technologies). Various ratios of pcDNA\textsuperscript{TM}6/TR (μg) and pTREX\textsuperscript{TM}-DEST30-LacZ (μg) between 3:1 and 12:1 were tested, in the presence and absence of tetracycline, to establish the ratio required for optimal gene induction (Fig. 5.12). A low basal level of gene expression was desired in un-induced samples, combined with high expression in tetracycline-induced samples. For PC3 cells, a 9 μg:1 μg ratio was selected (Fig. 5.12 A). For DU145 cells, the 6 μg:1 μg ratio of TR to LacZ was found to result in the lowest baseline signal and the highest induction (Fig. 5.12 B).
Figure 5.12 Optimisation of tetracycline-inducible gene expression in PC3 and DU145 cells.

The control vector pTREX™-DEST30-LacZ was used for co-transfections for optimisation purposes, in combination with pcDNA™6/TR vector. The pTREX™-DEST30-LacZ provides expression of β-Galactosidase, a readily detectable enzyme using a colorimetric chlorophenol red-beta-D-galactopyranoside (CRPG) format. Various ratios of pcDNA™6/TR and pTREX™-DEST30-LacZ vector DNA were tested in co-transfections in PC3 (A) and DU145 cells (B), in order to assess which ratios provided optimal induction of gene expression. Ratios of 3:1, 6:1, 9:1 and 12:1 (µg of pcDNA™6/TR: µg of pTREX™-DEST30-LacZ) were employed in co-transfections of cells with Lipofectamine® LTX. The following day, cells were incubated with 1 mg/ml tetracycline for 24 hours. Whole cell lysates were collected and analysed for β-Galactosidase production using a CRPG assay. Absorbance readings were obtained in the PowerWave™ at 570 nm. The optimal ratios of DNA (µg of pcDNA™6/TR: µg of pTREX™-DEST30-LacZ) which provided the lowest un-induced expression and most efficient gene induction were 9:1 for PC3 and 6:1 for DU145. Cells transiently transfected with pTREX™-DEST30-LacZ alone were included as a positive control (LacZ). Error bars denote S.E.M. Results shown are representative of three separate cell populations.
To confirm the effectiveness of these parameters in producing controlled expression of AR protein, the optimised conditions were tested in co-transfection experiments using pTREX™-DEST30-AR and pcDNA™6/TR. In both DU145 and PC3 cells, co-transfection with these vectors at the optimised ratios provided a tight regulation of AR protein expression (Fig. 5.13). As observed via western blot, a low basal level of AR was expressed in un-induced samples, and a marked induction of AR protein was observed in tetracycline-induced samples (Fig. 5.13). In the interests of time and cost, investigations were then focussed on one PCa cell line. Comparing the efficiency of AR induction between the transfected DU145 and PC3 cell lines, fold induction was greater in DU145 than PC3 cells, with respective values of 5.2 versus 1.46 (Fig. 5.12). Accordingly, further investigations of inducible AR protein expression were conducted in DU145 cells.

![Figure 5.13 Confirmation of optimal DNA ratios for transient co-transfection in PC3 and DU145 cells.](image-url)

The optimal DNA ratios were used to co-transfect PC3 (A) and DU145 (B) cells with pTREX™-DEST30-AR and pcDNA™6/TR, prior to induction with tetracycline (1 mg/ml; 24 hours) and collection of whole cell lysates. Samples were probed for AR protein expression via western blot, demonstrating that the chosen conditions provide a tight regulation of AR protein expression in both PC3 and DU145 cells. Results shown are representative of three separate experiments.
5.2.2.3 Optimisation of Tetracycline Dose for AR Induction

The optimal dose of tetracycline required to induce AR protein expression in DU145 cells was then investigated. After co-transfection with pTREX™-DEST30-AR and pcDNA™6/TR, DU145 cells were incubated in tetracycline concentrations from 0.1-10 µg/ml in culture medium for 24 hours. Whole cell lysates were collected and analysed for AR protein expression via Western blot (Fig. 5.14). A dose-dependent increase in AR protein expression was observed in response to increasing doses of tetracycline (Fig. 5.14), with a concentration of 0.5-0.75 µg/ml effectively producing moderate levels of AR protein expression.

![Western blot showing AR protein expression](image)

**Figure 5.14 Optimisation of tetracycline dose for AR induction in DU145 cells.**

DU145 cells were co-transfected with the pTREX™-DEST30-AR and pcDNA™6/TR vectors, and incubated in the presence of tetracycline (0.1-10 µg/ml) for 24 hours the following day. Samples were collected and analysed via western blot for AR protein expression. A tetracycline concentration of 0.5-0.75 µg/ml was found to be sufficient for induction of AR expression. Relative band density was calculated based on the β-actin loading control. Results shown are representative of three separate experiments, with densitometry performed on representative data.

5.2.2.4 Optimisation of Transfection Efficiency in DU145 Cell Line

The experimental conditions in transient transfection assays required tight control in order to obtain reliable and consistent data. The parameters for transfection were addressed and optimised, and subsequently kept consistent across all future experiments.

To maximise the efficiency of transient transfections, conditions were optimised in DU145 cells. Different amounts of DNA (0.06-0.3 µg/well), Lipofectamine® LTX (0.06-1.5 µl/well), the confluence of cultures on the day of transfection (50-90%) and varying lengths of incubation with transfection reagent (4–16 hrs) were tested. Cells were the incubated in the presence of tetracycline (0.5 µg/ml), prior to analysis of AR protein expression via immunocytochemistry using an anti-AR antibody. Maximal transfection efficiency and minimal toxicity were achieved using an 80-90% culture confluence at the time of transfection, 7 hour incubation
with transfection reagent and a 3:1 ratio of Lipofectamine® LTX (µl) to DNA (µg). The difference in results obtained with the use of non-optimised (Fig. 5.15 A) and optimised (Fig. 5.15 B) transfection conditions are shown in Fig. 5.15. Whilst the transfection efficiency was improved by this optimisation, the proportion of AR-expressing cells remained very low, at a transfection efficiency of only 10-20%.

Figure 5.15 Optimisation of DU145 transfection efficiency.
Cells were co-transfected with pTREX™-DEST30-AR and pcDNA™6/TR according to the original manufacturer’s recommendations in (A), and under optimised conditions in (B). Optimisation of DNA amount, LTX transfection reagent volume, transfection incubation time resulted in enhanced transfection efficiency. Cultures were left to recover for 16 hours prior to stimulation with tetracycline (0.5 µg/ml) for 24 hours. Cells were fixed and stained for AR protein expression using an anti-AR monoclonal antibody (shown in green) and Hoechst nuclear stain (shown in red). Despite achieving an improvement in transfection efficiency using these parameters, the number of cells displaying inducible expression of AR protein remained low under optimised conditions (B). Scale bar = 50µm.

To minimise variation between batches of transient transfectants, bulk transfections were performed in a larger vessel format (75cm² tissue culture flask). Cultures were left to recover for 16 hours in complete medium at 37°C, and harvested for re-seeding into 12-well plates. The next day, individual wells were treated with 0.1-1 µg/ml tetracycline (+/-10 nM DHT) for 24 hours prior to experiments. This format was kept consistent across assays.
5.2.3 The Effect of AR Expression on CXCR4

After establishing that AR expression was effectively induced in a dose-dependent manner with tetracycline after co-transfection with pTREX™-DEST30-AR and pcDNA™6/TR, the optimised system was used to investigate the effect of androgens on CXCR4 protein expression. Interestingly, the level of CXCR4 protein was enhanced after tetracycline-induction of AR protein expression in a dose-dependent manner (Fig. 5.16). This effect was specific to cultures co-transfected with pTREX™-DEST30-AR and pcDNA™6/TR; tetracycline had no impact on CXCR4 protein expression in DU145 cultures co-transfected with pTREX™-DEST30-empty and pcDNA™6/TR (Fig. 5.17).

![Figure 5.16](image1)

**Figure 5.16** Induction of AR up-regulated CXCR4 protein in co-transfected DU145 cells. DU145 cells were transfected with pTREX™-DEST30-Empty or pTREX™-DEST30-AR in combination with the pcDNA™6/TR vector, prior to induction with tetracycline (0.1-1 µg/ml) for 24 hours. Samples were collected and analysed via western blot for protein expression of AR, CXCR4 and the loading control β-Actin. Results shown are representative of three separate experiments. Density was expressed relative to the β-actin loading control, performed on representative data.

![Figure 5.17](image2)

**Figure 5.17** CXCR4 protein expression was unaffected by tetracycline in AR-negative DU145 cells. Cells were co-transfected with pTREX™-DEST30-empty and pcDNA™6/TR. Tetracycline (0.25-1 µg/ml) was applied the following day for 24 hours, prior to sample collection and analysis via western blot for protein expression of CXCR4 and AR. Results shown are representative of three separate experiments. Density was expressed relative to the β-actin loading control, performed on representative data.
Unfortunately, subsequent western blots of transfected cell populations demonstrated that the initial results shown in Fig. 5.16 could not be replicated (Fig. 5.18). Although the initial results were generated from three separate experiments, subsequent experiments (n=7) demonstrated that CXCR4 protein expression was not affected by induction of AR protein expression in transfected DU145 cells. Ultimately, due to a lack of reproducibility, no further investigations could be performed into the effect of androgens on CXCR4 in this system.

**Figure 5.18** CXCR4 protein expression was not affected by AR induction in subsequent DU145 co-transfection experiments.

DU145 cells were transfected with pTREX™-DEST30-Empty or pTREX™-DEST30-AR and with pCDNA™6/TR vector, prior to induction with tetracycline (0.75 µg/ml) for 24 hours. Samples were collected and analysed via western blot for protein expression of AR and CXCR4. Results shown are representative of seven separate experiments. Density was expressed in relation to the β-actin loading control, performed on representative data.
5.3 Discussion

In this chapter, the regulation of CXCR4 by androgens was investigated in androgen-insensitive PCa cells transfected with AR. Complications associated with the stable selection process resulted in a transient transfection approach being used for these studies with the tetracycline-inducible AR expression plasmid. Using a transient transfection system in DU145 cells, a positive association was observed between AR induction and CXCR4 protein expression. However, this response was not replicated in subsequent experiments. The difficulties in achieving reproducibility in this system of AR over-expression may link to a number of methodological reasons outlined below.

5.3.1 Stable Transfections

Stable transfectants were desired to provide an even expression and distribution of the AR protein in transfected cell populations, and a tetracycline-inducible construct was selected to permit control over the level of AR expression in a temporal fashion. However, despite the expression vector providing ligand-responsive AR expression in transient transfectants, no stable transfectants were successfully produced. Subsequent to antibiotic selection, AR protein expression was lost after 2-3 passages. Previous studies have shown that AR can be growth inhibitory or lethal in androgen-insensitive PCa cell lines (Shen et al. 2000); it is possible that this effect may have contributed to the loss of AR-positive cells after transfection. This occurred for cells populations transfected with either constitutive pTREX™-DEST30-AR, those co-transfected with pTREX™-DEST30-AR/ pcDNA™6/TR, and those sequentially transfected and selected with pcDNA™6/TR followed by pTREX™-DEST30-AR. It was likely that the loss of AR protein expression resulted from the rejection of the vector from cells over subsequent cell divisions, due to unsuccessful incorporation of the vector DNA into the cellular genome. Perhaps more success with generating stable transfectants may be achieved with alternative methods of transfection. These include electroporation of cells with the plasmid, or the use of a lentiviral transduction approach.
5.3.2 Transient Transfections

The results of the transient co-transfections using the pTREX™-DEST30-AR/pcDNA™6/TR vectors initially demonstrated results that were in contrast to the findings of previous studies. Akashi and colleagues (2006) demonstrated AR activity to negatively regulate CXCR4 expression and function in DU145 cells. Here, the enhanced CXCR4 protein expression in response to DHT was not observed in cells transfected with empty-vector after treatment with tetracycline, indicating that the effect was linked to induction of AR expression rather than the induction agent (tetracycline) itself.

Akashi and colleagues (2006) utilised stable transfectants of DU145 cells which were engineered to constitutively express AR mRNA and protein, which had been clonally selected based on AR expression levels. In the current study, the use of both transient transfections in combination with inducible AR expression system most likely contributed to these divergent results. Potential sources of these conflicting results are numerous, considering these initial results in the current study could not be replicated.

The likely reasons for the lack of reproducibility in the response reported here was the combined use of a transient transfection approach and a tetracycline-inducible system of expression regulation. Despite optimisation, the transient transfected populations displayed variable protein expression levels and low transfection efficiencies. Transient transfections can profoundly affect cell viability, and the length of culture recovery time post-transfection was limited by loss of AR protein expression at 48-72 hours post-transfection. Subsequently, experiments were performed on populations of cells that were considerably stressed in the time period immediately leading up to assays. In addition, the use of a tetracycline-inducible system meant that effective regulation of AR expression required cultures to be successfully co-transfected with both the pTREX™-DEST30-AR and pcDNA™6/TR vectors. This factor further reduced the population of cells per well that successfully displayed expression of inducible AR. Furthermore, not all of these cells respond to tetracycline induction. When considered together, all of these factors dramatically reduced the population of cells that could be used to analyse the impact on AR expression on chemokine receptor regulation.
Whilst there were indeed some advantages to using an inducible expression system in mammalian cells, this process was accompanied by complications that make the results more complex to interpret. The effect of the tetracycline itself on the system and the associated consequences for cell metabolism, behaviour and protein expression are not entirely known. It is known, however, that tetracycline and its derivatives can inhibit the function of MMPs at the level of mRNA expression and enzymatic activity (Shlopov 2001; Steinmeyer 1998; Vidal 2007). MMPs are known to play a role in cancer cell invasion and impact on invasive phenotype of tumour cells (Roomi 2010).

Although these results were not conclusive and further studies are required, there was an indication that androgens and the AR can exert regulation over the chemokine network. Accordingly, regulation of this central pathway may have implications for expanding the role of the AR pathway in regulating tumour cell invasion and metastasis during PCa progression.
6 CHAPTER SIX: THE FUNCTION AND REGULATION OF CXCR7 IN PCa CELLS
The results reported in this chapter have been published in the following journal article: *Chemokine receptor expression on integrin-mediated stellate projections of prostate cancer cells in 3D culture*. Kiss, D.L. Windus, L.C. Avery, V.M. Cytokine. August 2013. doi: 10.1016/j.cyto.2013.07.012. [Epub ahead of print]

### 6.1 Introduction

CXCR7 mediates organ morphogenesis, in addition to cell migration and cell positioning within the CNS during embryonic development (Dambly-Chaudiere *et al.* 2007; Sierro *et al.* 2007; Srinivasan *et al.* 2009; Valentin *et al.* 2007; Wang *et al.* 2012). CXCR7 expression has also been associated with tumour development and progression (Burns *et al.* 2006; Maio *et al.* 2007; Monnier 2012; Wang *et al.* 2008a; Xu 2011). Although CXCR7 is known to bind to both SDF-1α and ITAC, the receptor displayed a 10-fold lower affinity for ITAC when compared to SDF-1α. ITAC can also bind to CXCR3 (Shimizu *et al.* 2000), and as detailed previously, SDF-1α can also bind to CXCR4. CXCR7 can also elicit responses either by itself, or in combination with CXCR4, due to its ability to form a heterodimer with CXCR4 (Levoye *et al.* 2009).

As outlined in previous chapters, the SDF-1α/CXCR4 axis is well-established to promote the metastatic process of PCa; however, CXCR7 has also been found to regulate PCa cell survival and invasion (Wang *et al.* 2008a). *In vivo* prostate tumour biopsies showed a pattern where CXCR7 expression increases with invasive grade, as previously reported for CXCR4 (Sun 2003; Wang *et al.* 2008a). Wang and colleagues (2008a) have indicated a role for CXCR7 in PCa cell adhesion, invasion and angiogenesis, although the mechanisms mediating these processes are currently unknown. Whilst over-expression of CXCR4 can down-regulate CXCR7 expression in PCa cells (Wang *et al.* 2008a), CXCR4 antagonists interacted with and stimulated CXCR7 activity *in vitro* (Gravel *et al.* 2010; Kalatskaya *et al.* 2009). As such, these reports demonstrate that regulation of CXCR4 can impact on the expression and functionality of CXCR7. Further elucidation of this relationship is required to more fully understand how they regulate tumour growth and contribute to PCa progression.
The regulation of CXCR7 and CXCR4 also must be considered in the context of the tumour niche, as the progression of PCa is also facilitated by interactions of the tumour cells with their surrounding microenvironment (Clarke et al. 2009). The culturing of PCa cells in a 3D environment in vitro has previously been shown to alter gene and protein expression, and to induce altered morphological phenotypes (Harma et al. 2010). For instance, the PC3 cell line forms a stellate morphology in 3D culture, in which cellular extensions protrude out into the surrounding ECM (Harma et al. 2010). The association of cells with the ECM is mediated by adhesion proteins called integrins, and their movement through the ECM is permitted by expression of matrix-degrading enzymes such as MMPs (Clarke et al. 2009). The expression of integrin β1 on tumour cells can mediate an association with laminin in the extracellular matrix, contributing towards tumour cell invasion and metastasis (Belkin et al. 2000; Castronovo 1993). MMP-11 was up-regulated in cancer compared to normal epithelium, and was associated with tumour invasion and correlates with poorly differentiated tumours, metastatic events and poor prognosis (Andarawewa et al. 2003; Basset et al. 1997; Cheng et al. 2010; Masson et al. 1998; Noel et al. 2000).

The aim of this chapter was to characterise the expression and function of CXCR7 protein in PCa, using the more physiologically relevant in vitro 3D culture model. The regulation of CXCR7 was investigated in relation to CXCR4, through studying protein expression, localisation, growth regulation, and their association with the surrounding ECM in 3D culture. The results reported here indicate that the expression of CXCR7 and CXCR4 protein were linked with a more invasive morphology in 3D culture in vitro.
6.2 Results

6.2.1 CXCR7 in 2D Cultures of PCa Cells

6.2.1.1 CXCR7 Protein Expression in 2D

The expression of CXCR7 protein was assessed in conjunction with CXCR4 in the PCa cell lines DU145, PC3 and LNCaP. Higher expression of CXCR7 was previously associated with a more invasive PCa grade in vivo (Wang et al. 2008a). To investigate whether this finding was reflected in a higher expression of CXCR7 in more invasive PCa cell lines in vitro, comparative Western blots were performed. Interestingly, the less invasive LNCaP PCa cell line expressed comparable levels of CXCR7 and CXCR4 protein to PC3 and DU145 cells (Fig. 6.1).

![Figure 6.1 The expression of CXCR7 and CXCR4 protein in 2D cultures of PCa cells.](image)

Whole cell lysates of DU145, PC3 and LNCaP cultures were probed for CXCR7 and CXCR4 protein expression via Western blot. All cell lines tested were found to co-express CXCR7 and CXCR4. Densitometry was expressed relative to the loading control β-actin. Results shown are representative of three separate experiments, with densitometry performed on representative data.

6.2.1.2 Confirmation of Inhibitory Antibody Binding

The functions of CXCR7 and CXCR4 were investigated in PCa cells through the utilisation of function-blocking monoclonal antibodies in culture. Firstly, it was necessary to confirm that the CXCR7 and CXCR4 function blocking antibodies were binding to their specific targets in vitro. To this end, immunocytochemistry was utilised to study the localisation of receptors, before and after treatment with their shared ligand, SDF-1α, in 2D culture. LNCaP cells were employed for this purpose due to their strong co-expression of both CXCR7 and CXCR4 protein. In
controls, CXCR7 was observed as diffuse staining both at the cell membrane and within the cytoplasm (Fig. 6.2 A-A’). SDF-1α treatment (30 ng/ml; 40 minutes) induced the formation of punctuate structures, consistent with receptor internalisation (arrow, Fig. 6.2 B-B’). The CXCR4 antibody used for function blocking experiments was confirmed to specifically detect ligand-responsive CXCR4 in LNCaP via immunocytochemistry, as reported in Chapter 4 (Fig. 4.1 B-C).

Figure 6.2 The CXCR7 inhibitory antibody binds to its target receptor in 2D culture.

LNCaP cells were cultured in 2D, and either left untreated or stimulated with SDF-1α (30 ng/ml) for 40 minutes. Cultures were fixed and analysed for CXCR7 protein expression via immunocytochemistry. Control cells showed diffuse CXCR7 staining on the cell membrane and in the cytoplasm (A, image magnified further in A’), and treatment with SDF-1α resulted in a receptor internalisation response (B, image magnified further in B’). Arrow indicates punctuate vesicular structures of internalised receptor. Results shown are representative of three separate experiments. Scale bar = 50 μm.
6.2.1.3 The Effect of Chemokine Receptor Inhibition on PCa Cell Proliferation

Due to previous reports describing the regulation of cell growth or survival by CXCR7 in PCa and BCa cell lines (Burns et al. 2006; Wang et al. 2008a), the role of endogenously expressed CXCR7 in PCa cell growth was investigated using an Alamar Blue™ cell growth assay.

The regulation of PCa cell growth by the CXCR7, CXCR4 and their ligands were investigated in two ways. Firstly, the effect of chemokine ligands on LNCaP, DU145 or PC3 cell growth was assessed. ITAC (CXCR7/CXCR3 ligand), SDF-1α (CXCR7/CXCR4 ligand), or a combination of the two were applied to cultures, and cell growth was analysed by application of Alamar Blue™ after 3, 7, or 10 days of treatment. Treatment with these ligands, either separately or together, did not alter cell proliferation in any of the cell lines tested, under either FBS- or CS-FBS-supplemented culturing conditions (Fig. 6.3 A-C).
Figure 6.3 PCa cell line growth was unaffected by treatment with SDF-1α and/or ITAC. LNCaP (A), DU145 (B) or PC3 (C) cells were plated in culture medium containing 5% (v/v) FBS, and incubated with SDF-1α (50 ng/ml), ITAC (50 ng/ml) or both for up to 10 days. After 3, 7 and 10 days of treatment, Alamar Blue™ was added to wells at a final concentration of 10% (v/v). Plates were incubated at 37°C for 2 hours, and fluorescence readings were obtained on the EnVision® plate reader. Incubation with SDF-1α and/or ITAC for up to 10 days was not observed to alter the growth of PCa cell lines. Error bars denote S.E.M. Results shown are representative of three separate experiments, in triplicate. Results were analysed at day 10 using a t-test, and all were found to display no significant difference from cell growth in untreated (5% FBS) cell populations.
Secondly, the effect of receptor inhibition on cell growth was investigated using function-blocking monoclonal antibodies. CXCR7 shares ligand specificity with CXCR4 (SDF-1α) and CXCR3 (ITAC); therefore, inhibitory antibodies targeted against the CXCR4 and CXCR3 receptors were employed in parallel with the CXCR7 antibody (Fig. 6.4). These antibodies have been isolated and characterised based on their ability to bind and inhibit the function of their target receptor (R&D systems). The CXCR7 antibody (clone 9C4) used here has previously been demonstrated to display high specificity for CXCR7, and to neutralise CXCR7-induced cell migration to SDF-1α in T cell populations (Balabanian 2005). Incubation with anti-CXCR7, anti-CXCR4 or anti-CXCR3 function inhibiting antibodies in 5% (v/v) FBS was not observed to impact on the growth of LNCAP, DU145 or PC3 cell lines (Fig. 6.4).
Figure 6.4 The effects of CXCR7, CXCR4 or CXCR3 inhibitory antibodies on PCa cell proliferation.

LNCaP (A), DU145 (B) or PC3 (C) cells were plated in culture medium containing 5% (v/v) FBS, and incubated in the presence of anti-CXCR7, anti-CXCR4 or anti-CXCR3 antibodies (3-5 µg/ml) for up to 10 days. After 3, 7 and 10 days of treatment, Alamar Blue™ was added to wells at a final concentration of 5% (v/v) for 2 hours at 37°C. Fluorescence values were obtained by reading plates on the EnVision® plate reader. Addition of anti-CXCR7, anti-CXCR4 or anti-CXCR3 antibodies to cultures for up to 10 days was not observed to significantly alter cell growth in PCa cell lines. Error bars denote S.E.M. Results shown are representative of three separate experiments, in triplicate. Results were analysed at day 10 using a t-test, and all were found to display no significant difference from cell growth in untreated (5% FBS) cell populations.
6.2.1.4 Regulation of LNCAP Cell Growth by CXCR7 in Depleted Culturing Conditions

Interestingly, during investigations into the proliferation of LNCaP cells in the presence of CS-FBS, it was revealed that a reduced rate of growth was observed in the presence of the function-inhibiting anti-CXCR7 antibody (Fig. 6.5). The culturing of LNCaP cells in medium supplemented with CS-FBS significantly reduced the basal proliferation rate of LNCaP cells when compared with FBS (P=0.0216, Fig. 6.5 A), indicating that these depleted conditions are sub-optimal for the growth of this cell line. Whilst no significant effect on LNCaP cell proliferation was observed when the anti-CXCR7 antibody was employed in 5% (v/v) FBS, a statistically significant inhibition of growth was observed in 5% CS-FBS (v/v) after 10 days of treatment (Fig. 6.5 A). The growth inhibition effect was statistically significant at lower doses of CXCR7 function-blocking antibody (Fig. 6.5 B). The significant impact of anti-CXCR7 under depleted conditions, when compared to its lack of impact under general assay conditions in the presence of 5% (v/v) complete FBS, indicates that CXCR7 function may be required for LNCaP cell growth under depleted conditions. An alternative explanation for this result may include a non-specific toxic effect of the anti-CXCR7 antibody, which may have been masked in the presence of complete FBS.
Chapter Six: The Function and Regulation of CXCR7 in PCa Cells

Figure 6.5 The CXCR7 inhibitory antibody reduced LNCaP cell growth rates in CS-FBS.

LNCaP cells were plated in 5% (v/v) FBS or 5% (v/v) CS-FBS, and incubated with anti-CXCR7 antibody (5 µg/ml in A, 1-10 µg/ml in B) for up to 10 days. Cell growth was assessed on days 3, 7 and 10 through application of Alamar Blue™ directly to wells at a final concentration of 5% (v/v). After incubation at 37°C for 2 hours, fluorescence readings were obtained in the EnVision® plate reader. A comparison of anti-CXCR7 antibody applied in 5% (v/v) FBS and 5% (v/v) CS-FBS is shown in (A). The effect of 1-10 µg/ml doses of anti-CXCR7 antibody on LNCaP cell proliferation when incubated in 5% CS-FBS is shown in (B). Incubation of LNCaP cells with anti-CXCR7 antibody in depleted conditions (culture medium supplemented with CS-FBS) resulted in a statistically significant inhibition of cell growth (A). In comparison, the use of this antibody in FBS was not observed to alter cell proliferation (A). The inhibition of cell growth by the anti-CXCR7 antibody in culture medium supplemented with CS-FBS was found to be statistically significant at concentrations of 5-10 µg/ml (B). An IgG1 isotype control (5 µg/ml) produced an identical curve to cultures in the absence of isotype control. Statistical analysis was performed using a paired t-test at day 10. Error bars denote S.E.M. Results shown are representative of three separate experiments.
6.2.2 CXCR7 Protein Expression in 3D culture

6.2.2.1 Expression of CXCR7 protein in 3D Cultures Compared to 2D

The regulation of CXCR7 and CXCR4 in 2D culture was then compared to that observed in PCa spheroids generated through 3D culture conditions. Three-dimensional culture stimulates changes to the expression of protein associated with growth, adhesion, invasion and migration (Harma 2010). Thus we next evaluated whether any significant changes in the regulation of CXCR7 and CXCR4 protein expression were evident in 3D cultures of PCa cells, when compared to their 2D counterparts.

Cells were seeded in 2D or 3D conditions and maintained for up to 10 days. Samples were collected and analysed via Western blot for CXCR7 and CXCR4 protein expression. In the LNCaP (Fig. 6.6 A) and DU145 (Fig. 6.6 B) cell lines, the expression levels of CXCR7 and CXCR4 proteins were consistent between 2D and 3D conditions, and incubation with SDF-1α did not affect receptor expression at the protein level. However for the PC3 cell line, a marked enhancement of CXCR7 protein expression was observed in 3D culture, and was accompanied by a corresponding increase in CXCR4 protein expression (Fig. 6.6 C).

![Figure 6.6 The expression of CXCR7 and CXCR4 protein in 2D and 3D cultures of PCa cells.](image)

LNCaP (A), DU145 (B) or PC3 (C) cells were cultured in 2D or 3D conditions for 10 days, in the absence or presence of SDF-1α (50 ng/ml). Whole cell lysates were collected and analysed for CXCR7 and CXCR4 protein expression via Western blot. A significant increase in CXCR7 and CXCR4 protein expression was observed in 3D cultures of PC3 cells when compared to 2D culture (C). The expressions of these receptors in LNCaP (A) and DU145 (B) cells were found to be consistent between 2D and 3D cultures. Density of bands was expressed as relative to β-actin loading control. Results shown are representative of three separate experiments, with densitometry performed on representative data.
6.2.2.2 Chemokine Receptor Expression During PC3 Spheroid Development

The expression of CXCR7 and CXCR4 protein were then investigated over time in culture. Harma and colleagues (2010) have shown that the expression of genes associated with cell adhesion, invasion/metastasis, ECM turnover and mesenchymal markers were prominently altered after 6 days in 3D culture (Harma et al. 2010). Thus, it was of interest to ascertain the timeframe of this up-regulation in PC3 spheroid development. As spheroid size increased over time (Fig. 6.7 A), a substantial up-regulation of both CXCR7 and CXCR4 receptor protein expression was evident in PC3 cultures, particularly at days 7-10 of spheroid development (Fig. 6.7 B).

Figure 6.7 Expression of CXCR7 and CXCR4 protein over time in 3D cultures of PC3 cells.
DIC were obtained of PC3 cells grown as a 2D monolayer, and after 3, 7 and 10 days in 3D culture (A). Scale bar = 60 µm. Whole cell lysates were collected and analysed for CXCR7 and CXCR4 protein expression via Western blot (B). An increase in CXCR7 and CXCR4 protein expression were observed in PC3 cells after 7-10 days in 3D culture. Density of bands was calculated relative to β-actin loading control. Results shown are representative of three separate experiments, with densitometry performed on representative data.
Visual inspection of cultures at days 7-10 revealed that more prominent populations of stellate projections were present at these time-points (Fig. 6.7 A). To quantify stellate projection number and length, DIC images were obtained using the IN Cell 2000 for analysis at days 3, 5, 7 and 10 (Fig. 6.8 A). Due to the size PC3 spheroids and length of their extensions in the later stages of development, quantification of spheroid size and stellate projections was undertaken using a merged image of four fields of view, which was generated using IN Cell Developer software. Each image then contained an average of 15 spheroids, and from this the number and length of stellate projections were quantified using AxioVision LE software. The quantified data demonstrated a statistically significant increase in stellate projections at days 7-10, in terms of both the number of projections (Fig. 6.8 C) and their average length (Fig. 6.8 D).
Figure 6.8  The formation of spheroids in 3D cultures of PC3 cells over time.

PC3 cells were seeded in 2D or 3D and maintained in culture for up to 10 days. DIC images of cultures were obtained on days 3, 5, 7 and 10 using the IN Cell 2000, and stitched together from four adjacent fields of view per well using IN Cell Developer software (A). Scale bar = 500 µm. From these images, the number and length of stellate projections were measured using AxioVision LE software (B). Scale bar = 200 µm. The number of projections per spheroid (C) and the length of projections (D) were observed to increase over time in culture. Statistical analysis was performed using a paired t-test. Error bars denote S.E.M. n=44-60. Results shown are representative of four separate experiments.
6.2.2.3 Protein Expression of Integrin β1, MMP-11 and β-Laminin in PC3 Cultures

Next, the mechanisms through which stellate projections extended into the surrounding Matrigel were investigated by probing for the expression of proteins associated with cancer cell invasion. In particular, the expression of MMP-11, Integrin β1 and β-Laminin have been previously associated with tumour invasion and metastasis in vivo (Andarawewa et al. 2003; Basset et al. 1997; Cheng et al. 2010; Masson et al. 1998; Noel et al. 2000). A high protein expression of MMP-11 was observed on stellate projections (Fig. 6.9 A). Integrin β1 is known to mediate interactions with laminins to contribute towards tumour invasion (Belkin et al. 2000; Castronovo 1993). Similarly, integrin β1 protein expression was observed primarily on stellate projections and the periphery of the spheroid (Fig. 6.9 B), indicating a strong association with the ECM (Harma et al. 2010). A distinct expression of β-laminin protein was visualised on the periphery of the cell mass, extending out into the adjacent ECM (Fig. 6.9 C). The layer of β-Laminin may indicate that cells were actively secreting a layer of basal lamina from these stellate projections. Collectively, these results illustrate that stellate projections were actively expressing proteins associated with the process of cancer cell invasion. The presence of those PC3 stellate structures from days 7-10 may be indicative of a more invasive morphology in vitro.
Figure 6.9 Expression of integrin β1, MMP-11 and β-Laminin proteins in PC3 stellate projections.

PC3 cells were cultured in 3D for 7 days, fixed and processed via immunocytochemistry using antibodies against MMP-11 (A), integrin β1 (B) and β-Laminin (C). For panels A and C, MMP-11 and β-laminin are shown in red, F-actin staining by phalloidin in green and Hoechst nuclear stain in blue. In panel B, integrin β1 staining shown in green, F-actin staining by phalloidin in red and Hoechst nuclear stain in blue. The protein expression of MMP-11, integrin β1, and β-Laminin were up-regulated in the stellate projections of PC3 cells in 3D culture. Arrow indicates localisation in stellate projections. Scale bar = 50 µm. Results shown are representative of three separate experiments.
6.2.2.4 Expression of CXCR7 Protein in PC3 Stellate Projections

The observation of CXCR7 and CXCR4 protein up-regulation at later stages (days 7-10) of PC3 spheroid development prompted an investigation into whether the expression of these receptors were associated with stellate projections. The localisations of both CXCR7 and CXCR4 proteins were assessed via immunocytochemistry to determine receptor distribution in 3D culture (Fig. 6.10-6.11). CXCR7 was predominantly expressed on the stellate projections radiating out from the spheroid mass (arrow, Fig. 6.10 A), and a marked absence of protein expression was observed in the centre of the mass itself (Fig. 6.10 A).

In order to further verify the distribution pattern of CXCR7 and CXCR4 protein in PC3 cultures, 3D images of spheroids were reconstructed from 120 confocal z planes. Using these images, the distribution of receptor protein expression was verified throughout the spheroid mass. Consistent with a single plane, confocal images revealed that CXCR7 protein expression was localised to the stellate structures (arrow, Fig. 6.10 B), with minimal expression observed in the centre of the spheroid mass.
Figure 6.10 The expression of CXCR7 protein in stellate projections of PC3 cells in 3D culture.

PC3 cells were cultured in 3D for 7 days, fixed and processed for CXCR7 protein expression by immunocytochemistry. CXCR7 antibody staining is shown in green, phalloidin staining of F-actin in red and Hoechst staining of nuclei in blue. A single confocal slice of CXCR7 staining of a PC3 spheroid is shown in (A). Scale bar = 100 μm. A 3D reconstructed Z-stack of 120 confocal slices across a PC3 spheroid labelled with anti-CXCR7 antibody is shown in (B). Scale bar = 50 μm. Arrow indicates localisation of CXCR7 in stellate projections. Results shown are representative of three separate experiments.
Similarly, CXCR4 protein expression in PC3 cultures was also predominantly localised to stellate structures (arrow, Fig. 6.11 A), and a reconstructed z-stack revealed this distribution to be consistent throughout the spheroid (arrow, Fig. 6.11 B).

Figure 6.11 The expression of CXCR4 protein in PC3 stellate projections in 3D culture. PC3 cells were cultured in 3D for 7 days, fixed and CXCR4 protein expression was probed via immunocytochemistry. CXCR4 antibody staining is shown in green, phalloidin staining of F-actin in red and Hoechst staining of nuclei in blue. A single confocal slice of CXCR4 staining of a PC3 spheroid is shown in (A). Scale bar = 100 µm. A 3D reconstructed Z-stack of 120 confocal slices across a PC3 spheroid labelled with anti-CXCR4 antibody is shown in (B). Scale bar = 50 µm. Arrow indicates localisation of CXCR4 in stellate projections. Results shown are representative of three separate experiments.
This receptor distribution was further verified by co-labelling for CXCR7 and CXCR4 protein expression (Fig. 6.12). A distinct co-localisation of CXCR7 and CXCR4 proteins were observed on stellate projections (arrow, Fig. 6.12), demonstrating their expression patterns to be heavily associated with each other.

![Figure 6.12 Co-localisation of CXCR7 and CXCR4 protein expression in stellate projections of PC3 cells.](image)

PC3 cells were cultured in 3D for 7 days prior to fixation and processing via immunocytochemistry for protein expression of CXCR4 (A) and CXCR7 (A’). CXCR4 antibody staining is shown in green, CXCR7 antibody staining is shown in red, and Hoechst staining of nuclei in blue. An overlay of CXCR7 and CXCR4 staining illustrated a co-localisation of the receptors (A’’), particularly in stellate projections (arrow). Scale bar = 100 µm. Results shown are representative of three separate experiments.
To study the role of CXCR7 and CXCR4 protein up-regulation in PC3 cells in 3D, and their association with stellate projections, the regulation of PC3 spheroid morphology by CXCR7 and CXCR4 was investigated.

6.2.2.5 The Effect of Chemokine Receptor Inhibition on 3D Cultures of PC3 Cells

To investigate the potential significance of enhanced CXCR7 and CXCR4 protein expression in 3D cultures of PC3 cells, the effects of receptor inhibition on the spheroid growth and development were studied.

6.2.2.5.1 Spheroid Growth

PC3 cells were plated in 384-well plates, and cultured in 3D for up to 10 days in the presence or absence of chemokine receptor inhibitors (anti-CXCR4 or anti-CXCR7 antibody, CXCR4 antagonist AMD3100, or isotype controls). Spheroid growth was assessed using an Alamar Blue™ assay. Incubation with any of these inhibitors for up to 10 days did not alter the growth of PC3 spheroids in 3D culture (Fig. 6.13).

![Figure 6.13 Inhibition of CXCR7 or CXCR4 did not impact on PC3 spheroid growth.](image)

PC3 cells were cultured in 3D for up to 10 days in the presence of either 5% (v/v) FBS with the addition of anti-CXCR7, anti-CXCR4 or anti-CXCR3 function-inhibiting antibodies (3-5 µg/ml). After 3, 7 or 10 days of treatment, cell growth was assessed by addition of Alamar Blue™, and incubated for 2 hours at 37°C, prior to obtaining fluorescence readings on the EnVision® plate reader. Incubation with anti-CXCR7, anti-CXCR4 or anti-CXCR3 antibodies were not observed to affect PC3 cell proliferation in 3D culture. Error bars denote S.E.M. Results shown are representative of three separate experiments.
6.2.2.5.2 Development of Stellate Projections in Culture

To further elucidate the role of CXCR7 and CXCR4 protein up-regulation in PC3 stellate projections, the effect of receptor inhibition on 3D cultures were investigated. Considering that the expression of CXCR7 and CXCR4 proteins were heavily associated with stellate projections in cultures of PC3 cells in 3D, we then asked if their function was required to maintain the stellate morphology observed in 3D cultures. DIC images were obtained using the IN Cell 2000, on days 3, 5, 7 and 10 of treatment with anti-CXCR7, anti-CXCR4 function-inhibiting antibodies and/ or the CXCR4 antagonist AMD3100. Four adjacent fields of view were combined into one image for subsequent analysis of projection number and size, using AxioVision LE (Fig. 6.14 A). The spheroid images from day 10 have been shown in Fig. 6.14 A to more clearly demonstrate the lack of treatment impact on morphology. Firstly, image analysis showed that inhibition of either CXCR7 or CXCR4 did not alter spheroid diameter (Fig. 6.14 B), which, combined with results from the Fig. 6.13, demonstrated that receptor function was not required for growth of PC3 spheroids in 3D culture. Secondly, analysis of these images for stellate projection number (Fig. 6.14 C) revealed that inhibition of either CXCR7 or CXCR4 did not affect the number of stellate projections in culture. Therefore, these data showed that CXCR7 or CXCR4 function was not required for the formation or maintenance of stellate projections in PC3 cells.
Figure 6.14 Inhibition of CXCR7 and/or CXCR4 did not impact on PC3 stellate projections in 3D culture.

PC3 cells were incubated in the presence of anti-CXCR4, anti-CXCR7 antibody and/or the CXCR4 antagonist AMD3100 (all 5 µg/ml) for 10 days in 3D culture. DIC images were obtained on the IN Cell 2000 on day 10 (A). Grey lines indicate the points at which images were “stitched” together and compiled using IN Cell Developer software. Scale bar = 200 µm. Images were used for analysis of spheroid size (B) and stellate projection number (B) with AxioVision LE software. No statistically significant effect on the development of stellate projections was observed in the presence of anti-CXCR4, anti-CXCR7 antibody and/or CXCR4 antagonist AMD3100. Statistical analysis was performed using a paired t-test. Error bars denote S.E.M. n=44-60. Results shown are representative of three separate experiments.
6.2.2.6 Loss of PC3 Stellate Morphology after Functional Inhibition of Integrin β1

Due to the strong association of integrin β1 expression with the stellate projections of PC3 cells, next it was investigated whether the function of this protein was required to maintain the stellate morphology. Cultures were incubated with isotype control or function blocking anti-integrin β1 antibody for 7 days in culture, at which point the formation of stellate projections was apparent (Fig. 6.15 A). Inhibition of integrin β1 resulted in a distinct change to PC3 spheroid morphology (Fig. 6.15 B). As viewed via DIC and immunocytochemistry, a distinct lack of stellate projections were observed in the inhibited cultures. Thus, it was evident that the function of integrin β1 was required for formation and maintenance of the stellate morphology in PC3 cells.

Figure 6.15 Functional inhibition of integrin β1 altered PC3 spheroid morphology.

DIC images of PC3 cells cultured in 3D were obtained, after incubating cultures in the presence of isotype antibody control (A) or function inhibiting anti-integrin β1 antibody (B) for 7 days. Immunocytochemical analysis of spheroid morphology in integrin β1-inhibited cultures are shown in (B’), with phalloidin staining of F-actin demonstrated in green, and Hoechst staining of nuclei in blue. Scale bar = 30 µm. Incubation with the anti-integrin β1 antibody during spheroid development inhibited the formation of stellate projections in PC3 spheroids. Results shown are representative of four separate experiments.
6.2.2.6.1 The Effect of Integrin β1 Inhibition on CXCR7 and CXCR4

The expression of CXCR7 and CXCR4 proteins were then studied in cultures incubated with the anti-integrin β1 antibody. Western blot analysis revealed that cultures incubated with the integrin β1 antibody displayed a reduction in CXCR7 protein expression when compared to isotype antibody controls, as observed via Western blot (Fig. 6.16 A). This finding was confirmed using immunocytochemistry (Fig. 6.16 B, C). The CXCR7 cytoplasmic staining observed in these images demonstrates that this receptor is localised to both the cytoplasm and cell membrane in its resting state. Cells incubated with isotype antibody control displayed CXCR7 protein expression in stellate projections (Fig. 6.16 B), whereas stellate projections were absent in cultures treated with anti-integrin β1 antibody, and CXCR7 protein expression was down-regulated (Fig. 6.16 C).
Figure 6.16 Inhibition of integrin β1 down-regulated CXCR7 protein expression in PC3 cells.

PC3 cells incubated in 3D culture for 7 days in the presence of isotype antibody control or anti-integrin β1 antibody. Cultures were lysed and probed for CXCR7 protein expression via Western blot (A). Density was expressed in relation to β-actin loading control. An anti-CXCR7 antibody was employed for immunocytochemistry studies (B-C). The protein expression of CXCR7 is shown for isotype antibody controls in (B), and in cultures incubated with anti-integrin β1 antibody in (C). CXCR7 staining is shown in red, and F-actin staining by phalloidin in green and Hoechst staining of nuclei in blue. Scale bar = 30 µm. Incubation with the anti-integrin β1 antibody during spheroid development inhibited the protein expression of CXCR7. Results shown are representative of three separate experiments, with densitometry performed on representative data.
Analysis of CXCR4 protein levels via Western blot demonstrated CXCR4 protein expression was reduced in cultures treated with the integrin β1 function blocking antibody (Fig. 6.17 A). The reduction in CXCR4 protein expression was also observed via immunocytochemistry using an anti-CXCR4 antibody. A comparison of cultures incubated with isotype control (Fig. 6.17 B) versus those incubated with anti-integrin β1 antibody (Fig. 6.17 C) illustrated a reduction in CXCR4 protein expression. Furthermore, CXCR4 protein localisation was affected by incubation with anti-integrin β1 antibody. Whilst isotype antibody controls displayed a diffuse cytoplasmic CXCR4 staining pattern, cultures incubated with anti-integrin β1 antibody displayed an enhanced membrane localisation of the receptor (Fig. 6.17 B-C).
Figure 6.17  CXCR4 protein expression was reduced after incubation with integrin β1 antibody in PC3 cells.

3D cultures of PC3 cells were incubated in the presence of isotype antibody control or anti-integrin β1 antibody for 7 days. Whole cell lysates were analysed for CXCR4 protein expression via Western blot (A). Density was calculated in relation to the β-actin loading control. Alternatively, an anti-CXCR4 antibody was used in immunocytochemistry studies. PC3 cells treated with isotype control displayed CXCR4 protein expression, particularly in stellate projections (B-B’). Incubation with the anti-integrin β1 antibody was observed to reduce CXCR4 protein expression (C-C’). CXCR4 staining is shown in red, F-actin staining of phalloidin in green and Hoechst staining of nuclei in blue. Incubation of PC3 cells with anti-integrin β1 antibody during spheroid development inhibited the expression of CXCR4 protein. Scale bar = 30 µm. Results shown are representative of three separate experiments, with densitometry performed on representative data.
These studies demonstrated that functional inhibition of integrin β1 resulted in a loss of the characteristic PC3 stellate morphology in 3D culture. The expression of CXCR7 and CXCR4 proteins, which we have previously shown to display a strong association with stellate projections, were down-regulated in these cultures. This indicates that, although the expression of CXCR7 and CXCR4 proteins were not required for development of the stellate morphology in PC3 cells, their expression may indirectly contribute towards invasive characteristics of PC3 cells via an interaction with integrin β1.
6.3 Discussion

In summary, the regulation of CXCR7 and CXCR4 was investigated in both 2D and 3D cultures of PCa cells. Both CXCR7 and CXCR4 are expressed in LNCaP, DU145 and PC3 cell lines in 2D culture. Inhibition of CXCR7 under depleted conditions was observed to impact on cell proliferation rates in the LNCaP cell line, whilst cells incubated under complete culturing conditions were unaffected. Here, it was demonstrated for the first time that 3D cultures of PC3 cells displayed an enhanced expression of both CXCR4 and CXCR7 proteins, in comparison to their 2D counterparts. The enhanced protein expression of these receptors in 3D culture was associated with stellate projections, which displayed substantial expression of proteins associated with tumour adhesion and invasion: Integrin β1, MMP-11 and β-laminin. This enhanced expression of CXCR7 and CXCR4 protein in the invasive stellate morphology of PC3 cells was consistent with the up-regulation of these receptors during progression to invasive PCa observed in vivo (Sun 2003; Wang et al. 2008a). Furthermore, the results reported here indicate that the stellate morphology of PC3 cells, and the associated protein expression of both CXCR7 and CXCR4, was mediated by integrin β1-mediated interactions with the surrounding ECM. Altogether, these results demonstrate that integrin β1 can profoundly affect tumour spheroid morphology and the expression of chemokine receptors, which are known to promote the invasion and progression of PCa.

6.3.1 CXCR7 Regulated LNCaP Cell Growth Rates Under Conditions of Stress

Investigations of CXCR7 in 2D cultures of PCa cell lines revealed a role for this receptor in regulation of LNCaP cell growth or survival under depleted conditions. The culturing of androgen-dependent LNCaP cells in CS-FBS reduced their basal proliferation rate significantly. The charcoal stripping process used to generate CS-FBS is known to reduce the levels of endogenous hormones including androgens and oestrogens in FBS (Eckert 1982; Sedelaar et al. 2009). LNCaP cells are dependent on androgens for their growth, and it was likely that the reduced levels of androgens in CS-FBS-supplemented culture medium resulted in the reduced rate of cell growth, placing these cultures under stress.
in depleted conditions. Employment of the anti-CXCR7 function blocking antibody under these conditions further inhibited the proliferation rate of LNCaP cells. To further clarify the specific nature of this inhibitory antibody effect on LNCaP cell growth, future studies can be carried out by addition of SDF-1α and/or ITAC to CXCR7-inhibited LNCaP cultures. This could demonstrate whether the addition of ligand can reverse the effect of the anti-CXCR7 antibody on LNCaP growth by competing for CXCR7 binding sites. Such findings could assist in confirming the specificity of this response for CXCR7.

This observation of LNCaP growth inhibition in the presence of the anti-CXCR7 antibody was consistent with the role of CXCR7 in cell growth and survival under depleted conditions, reported in the MDA-MB-468 BCa cell line by Burns and colleagues (2006). Transfection of the MDA-MB-468 cell line with CXCR7 enhanced cell survival in a low-serum (1% FBS) environment when compared to controls. Here, the cell growth assays indicate that endogenous CXCR7 protein may influence LNCaP cell growth cultured under depleted conditions. At this stage, it is unclear as to whether cell survival or cell proliferation was responsible for this observation in PCa cells. Further research is required to determine the mechanism of this effect, and consequences of this regulation in LNCaP cells. These preliminary findings may indicate that CXCR7 function becomes more important to tumour cell growth or survival under conditions of stress; such a finding may be relevant to the targeting of CXCR7 function when PCa cells under stress, such as in the hypoxic core of a PCa tumour mass.

Typically, it is the stimulation of chemokine receptors with their chemokine ligands that results in a functional response. Here, it was found that stimulation of PCa cells with CXCR7 and/or CXCR4 with exogenous SDF-1α and/or ITAC did not impact on cell proliferation in PCa cell lines. However, blocking the basal activity of CXCR7 in the absence of exogenous ligand impacted on cell growth in LNCaP cells under CS-FBS conditions. This indicated that the basal activity of CXCR7 can regulate LNCaP cell growth or survival under depleted conditions. It remains to be established whether this basal activity of CXCR7 may be a consequence of constitutive receptor activity or activation of the receptor by factors present endogenously in the culture medium.
This regulation of basal cell growth rate by endogenous CXCR7 activity, rather than ligand-stimulation, was consistent with previous studies. The reports of cell growth regulation by CXCR7 in tumour cells have been performed in systems of CXCR7 over-expression, or though siRNA inhibition of CXCR7 expression, rather than as a consequence of ligand stimulation. In particular, Burns and colleagues (2006) showed that MDA-MBA-435 BCa cells transfected with CXCR7 proliferate more rapidly than controls. In MCF7 cells, Boudot and colleagues (2011) reported an increase in basal cell growth rates after transient transfections with CXCR7. In PCa cells, Wang and colleagues (2008a) demonstrated that over-expression of CXCR7 enhanced the basal cell growth rates in PC3 and LNCaP C4-2B cell lines. Indeed, inhibition of CXCR7 function with a siRNA inhibited both PCa and BCa tumour growth in mice, in comparison to controls (Burns et al. 2006; Wang et al. 2008a). The effect of CXCR7 inhibition using specific CXCR7-targeted shRNA/siRNA, and studies of CXCR7 over-expression, could also be necessary in future work, to confirm the results observed in the current study.

Further investigations are required to determine whether CXCR7 is required specifically for cell growth or cell survival, to contribute to the regulation of basal cell growth rates in PCa cells. This could be investigated through assessment of cell viability, or through analysis for markers for apoptosis or necrosis, versus markers for cell proliferation. As mentioned above, this could also be performed in cells transfected with CXCR7-specific shRNA/siRNA to increase confidence in these results obtained with the neutralising CXCR7 antibody. If the mechanism and consequences of this effect can be further clarified in PCa cells, this may provide evidence to suggest that combinatorial administration of CXCR7 inhibitors with other anti-cancer treatments may potentiate the effectiveness of traditional therapies. Specifically, further investigations are required to determine whether CXCR7 inhibition during hormone deprivation therapy may potentiate the effectiveness of ADT in halting tumour growth during the androgen-sensitive, androgen-dependent stage of PCa.
6.3.2 Stellate Projections Were Rich in the Expression of Pro-Invasive Proteins

The characteristic PC3 stellate morphology in 3D culture was accompanied by the expression of integrin β1, β-laminin and MMP-11 proteins. Integrin β1 expression on these structures was consistent with findings by Harma and colleagues (2010). Integrin β1 is known to mediate cancer cell adhesion, particularly to fibronectin and laminin in the ECM (Harma et al. 2010; Kulbe et al. 2004). The emergence of a β-Laminin layer surrounding the stellate projections of PC3 cells may further promote the engagement of integrins with the ECM (Harma et al. 2010). The protein expression of MMP-11 at stellate projections indicates that these cell populations are actively invading through the surrounding Matrigel. Collectively, the expressions of these pro-invasive proteins are consistent with the stellate PC3 morphology representing a more invasive phenotype in vitro.

6.3.3 CXCR7 and CXCR4 Protein Expression in Stellate Projections

The strong up-regulation of CXCR7 and CXCR4 protein observed in 3D cultures of PC3 cells may be a consequence of cross-talk with other cellular pathways that encourage growth and invasion. Harma and colleagues (2010) have demonstrated that 3D culture triggers changes to gene expression which corresponded with particular morphologies. In that study, the emergence of the stellate phenotype was associated with genes regulating cell adhesion, cell-cell contact, invasion/metastasis and ECM turnover and mesenchymal markers (Harma et al. 2010). The development of the stellate morphology was described as an invasive transformation of PC3 cells in vitro, accompanied by enhanced activity through signalling pathways such as PI3K, Akt and JAK-STAT (Harma et al. 2010). Both pro-invasive protein expression and enhanced signalling pathway activities can up-regulate chemokine receptor expression in vitro (Busillo et al. 2007; Li et al. 2004). Therefore, enhanced activity through these pathways during the emergence of the stellate phenotype may result in enhanced expression of CXCR7 and CXCR4 proteins.

The strong up-regulation of both CXCR7 and CXCR4 protein at 3D cultures of PC3 cells was accompanied by a co-localisation of these proteins at stellate projections. This demonstrates that both receptors can be concurrently up-
regulated, in contrast to the inverse association reported previously for these receptors in systems of over-expression (Wang et al. 2008a). Specifically, Wang and colleagues (2008a) demonstrated that transfection of the LNCaP C4-2B subline and PC3 cells with CXCR4 resulted in a down-regulation of CXCR7 protein expression in 2D culture (Wang et al. 2008a). The positive relationship between CXCR7 and CXCR4 observed in the current study may be due to the study of endogenous receptor regulation, as opposed to the findings of the previous studies of over-expression (Wang et al. 2008a). Potentially, over-expression may exert an abnormal feedback response in vitro, due to saturation of cellular machinery with the over-expression of one protein. Certainly the observations of concurrent up-regulation that were reported in the current study were more consistent with the enhanced expression of both CXCR7 and CXCR4 with PCa invasive grade in vivo (Akashi et al. 2008; Mochizuki et al. 2004; Wang et al. 2008a).

6.3.4 **CXCR7 and CXCR4 Function Do Not Mediate PC3 Stellate Morphology**

The up-regulation of both CXCR7 and CXCR4 protein at stellate projections in 3D culture prompted an investigation into the association between the stellate morphology and the protein expression of these receptors. CXCR7 and/or CXCR4 function were not required for the growth and development of PC3 spheroids, or the development of stellate projections. The results presented here suggested that the enhanced expressions of CXCR7 and CXCR4 were a consequence of stellate morphology.

6.3.5 **Development of Stellate Projections Were Mediated by Integrin β1**

The interaction of cells with the ECM via integrin β1 activity was shown to mediate the stellate morphology of PC3 cells in 3D culture. Inhibition of integrin β1 with a function blocking antibody was found to dramatically influence the development of stellate morphology by inhibiting the formation of projections. This demonstrated that the interaction of the PCa cells with the surrounding ECM can play an important role in maintaining the structure and morphology of PCa spheroids in vitro.
6.3.6 Regulation of CXCR4 and CXCR7 Protein Expression via Integrin β1

Here, it was demonstrated that expression of CXCR7 and CXCR4 proteins were reduced by functional inhibition of integrin β1 protein. It was likely that the loss of stellate morphology under the inhibited condition resulted in reduced receptor protein expression. The inhibition of integrin β1 also altered the localisation of CXCR4 protein, from a predominantly cytoplasmic localisation to a membrane-associated protein expression pattern. It is known that CXCR4 present on the cell membrane is capable of binding and mediating functional responses to extracellular ligand (Busillo et al. 2007; Signoret et al. 1997). Perhaps this reflects an impact of integrin β1 inhibition on CXCR4 functionality, a factor which requires further investigation.

Collectively, it was evident that integrin β1 can profoundly impact on PC3 spheroid development and morphology. Furthermore, the morphological changes observed within inhibited cultures were accompanied by modulated protein expression of the CXCR7 and CXCR4 receptors, which were associated with the more invasive, stellate morphology of these cells. Further studies are required to elucidate the impact of this regulation on tumour cell phenotype in vitro and in vivo. This may indicate a combined role for CXCR7 and CXCR4 in regulating the invasive morphology of PCa cells through integrin-mediated interactions with the ECM. Subject to further investigation, these findings may indicate that combined blockade of both the CXCR7 and CXCR4 receptors may be more effective in controlling PCa tumour growth and progression than monotherapies targeted separately to one receptor.
CHAPTER SEVEN: CONCLUSIONS AND FUTURE DIRECTIONS
The contributions of CXCR4 to the progression of PCa to metastasis have resulted in the status of this receptor as a therapeutic target (Ben-Baruch 2008; Sun 2003; Woodard 2011). The actions of CXCR4 which promote tumour metastasis, however, must be considered in relation to other factors present in the tumour microenvironment. In order to understand the complexity, implications and limitations of the potential for this chemokine receptor as a therapeutic target, further characterisation of the redundancy and regulation within the chemokine network is required.

Here, the mechanisms regulating CXCR4 protein expression and function in PCa cells were investigated, to further characterise the factors which exert influence over this pathway. Previous studies detailing androgenic regulation of CXCR4 in androgen-sensitive LNCaP cells prompted an investigation into whether this regulation could be observed in androgen-insensitive PCa cells (Frigo 2009). Tumour cell phenotype can also be influenced by the ECM and other factors which are present in the tumour microenvironment (Sung et al. 2007). The regulation of CXCR4 by the surrounding ECM was investigated using 3D culture, in conjunction with the alternative SDF-1α binding receptor, CXCR7. The contribution of integrin-ECM interactions to the regulation of these receptors was also assessed. When considered together, the results presented in this study indicate that a complex regulation network may exist between the ECM, CXCR7, CXCR4 and the AR in PCa cells.

7.1 AR and Chemokine Regulation in PCa: The Impact of 3D Culture

The presence of an ECM in 3D culture supported the endogenous re-expression of AR protein in PC3 cells. AR has been well-established to regulate the development and progression of PCa (Green 2012), and here, for the first time, it was shown that cell-ECM interactions can regulate AR protein expression in vitro. PC3 cells do not express AR in 2D culture, however in 3D culture it was clearly up-regulated; the critical role of the ECM in this observation was demonstrated when removal of PC3 cells from the 3D environment resulted in a loss of protein expression.

Furthermore, it was found that endogenously expressed AR protein in the androgen-insensitive PC3 cells displayed a differential regulation and function to that observed in the androgen-sensitive LNCaP cells. Whereas AR protein expression was maintained during signalling pathway inhibition assays in LNCaP cells, the expression of AR protein in PC3 cells was modulated by Src/MAPK pathway inhibition. This showed that endogenously re-expressed AR in 3D cultures of PC3 cells was regulated differently to
LNCaP cells. Analysis of AR functionality between these cell lines revealed that, whilst the AR protein expressed in LNCaP cells produced PSA and regulated cell growth, the AR in PC3 cells displayed altered functionality. The androgen-insensitive growth of this cell line was maintained in 3D culture, and stimulation of PC3 cells with DHT resulted in nuclear translocation of AR; however no PSA production was detected, indicating that the activation of AR protein in PC3 cells was not followed by transcription of its target gene, PSA. Measurement of PSA expression is widely used to measure AR activity in vitro (Jia et al. 2005), however to confidently determine whether AR is transcriptionally active in this cell line, measurement of additional AR-target genes are required. For example, AR reporter gene assays that rely upon transient transfections with luciferase constructs are not feasible for use in 3D culture due to spheroid penetration issues. Alternatively, the detection of target gene transcription at an mRNA level could be analysed via RT-PCR using primers for alternative AR target genes, such as TMPRSS2. Despite the requirement for further study, it was evident that the AR expressed in androgen-insensitive PC3 cells did not mirror the functional response of AR observed in androgen-sensitive LNCaP cells. This suggested that downstream activities of AR may vary between androgen-sensitive and androgen–insensitive PCa cells. Indeed, differences have been noted in the activation of PSA transcription by AR in the androgen-sensitive LNCaP cell line when compared to its androgen-insensitive subline LNCaP C4-2B (Jia et al. 2005). Although both cell lines produced detectable levels of PSA, the mechanism of PSA transcription in the C4-2B cell line was altered, requiring an intermediate mechanism rather than direct binding of the AR to the PSA promoter (Jia et al. 2005).

Here, androgen regulation of CXCR4 was observed in androgen-insensitive PC3 cells. This finding demonstrated that this aspect of CXCR4 regulation remained relevant to PCa cells which do rely on the presence of androgens for cell growth. Based on this initial finding, a role for androgens was indicated in the regulation of PCa progression through modulation of CXCR4. However, to determine the potential consequences of this regulation, further studies are necessary. For example, the effects of androgen deprivation on CXCR4 expression and function could be investigated. Androgen deprivation could be generated through culturing in CS-FBS rather than FBS for a period of 1-3 months. A comparison of CXCR4 expression and function between LNCaP and PC3 cells under these conditions may demonstrate whether the consequences of androgen starvation on CXCR4 display variation between androgen-
sensitive and androgen-insensitive PCa cells. The results of this experiment may indicate whether there could be value in targeting CXCR4 for therapeutic inhibition in PCa patients undergoing ADT, to slow progression to metastasis. Although the role of CXCR4 and AR in PCa progression must be considered in the context of other contributing factors within the tumour microenvironment, these findings may help to elucidate the potential mechanisms that influence the emergence of CRPC in PCa cells.

7.2 A Role for CXCR7 in Regulating PCa Cell Growth Rate in Depleted Culturing Conditions

The results reported here also demonstrated a role for CXCR7 in androgen-sensitive LNCaP cells regarding cell growth. Treatment of cells with an inhibitory CXCR7 antibody under depleted culturing conditions (CS-FBS) resulted in a significant reduction in LNCaP cell proliferation rates. By comparison, the same antibody had no effect on LNCaP cell proliferation in complete medium containing FBS. Further studies are now required to distinguish between decreased cell proliferation itself, and reduced cell survival. These two possibilities could be investigated using a cell viability assay, for example the Live/Dead fixable stains which are commercially available for analysis via flow cytometry (Life Technologies). To increase confidence in the results obtained using the neutralising CXCR7 antibody, cells will be transfected with CXCR7-specific shRNA/siRNA, to determine if similar results can be replicated using this approach.

The depletion of FBS with charcoal results in the removal of hormones including androgens and estrogens, as well as some growth factors, from the preparation (Eckert 1982; Ochi et al. 1973; Sedelaar et al. 2009). Further studies are required to determine whether the effect of CXCR7 inhibition on cell growth under these conditions was specific to a low-hormone environment, or due to a general stress response in the cell population as a result of growth factor removal. This could be investigated by performing an Alamar Blue™ proliferation assay in the presence of the inhibitory CXCR7 antibody with the addition of DHT, to determine if the reduced rate of growth in CXCR7-inhibited cultures is rescued in the presence of androgens. If the results of this experiment can confirm the cell growth inhibitory effect of the CXCR7 antibody can be rescued by androgens, this finding may have implications for potential therapeutic approaches to PCa. While patients are being treated with ADT, CXCR7 function may be required for PCa cell growth or survival; and perhaps targeting CXCR7 during ADT may potentiate the inhibition of PCa growth and progression.
Subject to the results of further investigations, the hypothesis could be tested \textit{in vivo}. A role for CXCR7 in tumour development has been previously demonstrated in the study performed by Wang and colleagues (2008a). The role of CXCR7 in tumour development and metastasis in relation to ADT could be studied using orthotopic xenograft mouse models (Lin et al. 2013). SCID mice injected with LNCaP cells transfected with CXCR7 siRNA or shRNA could be treated with ADT. Alternatively, SCID mice injected with untransfected LNCaP cells could be treated with ADT. After 4-5 weeks of growth and treatment, the number and volume of tumours could be measured, to determine whether this combination treatment results in reduced PCa tumour growth and/or a reduction in the numbers of metastatic deposits. The outcomes of this study could assist in determining whether CXCR7 function is important for PCa cell survival in low-hormone conditions.

7.3 Regulation between CXCR4, CXCR7 and the ECM in PCa cells

When the results reported in this thesis are considered together, it is evident that a relationship exists between AR, CXCR4, CXCR7 and integrin-mediated interactions of PCa cells with the ECM \textit{in vitro}. The culturing of PC3 cells in 3D matrices resulted in dramatic changes to the regulation of AR, CXCR4 and CXCR7. In particular, AR was shown to regulate CXCR4 protein expression, which in turn, was co-expressed with CXCR7 to contribute towards a more invasive morphology of PCa cells \textit{in vitro}. The development and maintenance of this morphology was dependent on integrin-mediated interactions with the ECM, which also regulated the expression of both CXCR4 and CXCR7 protein. The expression of these receptors in PCa cells has been associated with enhanced cell migration and invasion (Chinni et al. 2006; Wang et al. 2008a).

Further studies are required to determine the consequences of enhanced CXCR7 and CXCR4 expression in PCa cells. Cell invasion assays could be used to study the link between more invasive stellate morphology and capacity to undergo cell invasion. An \textit{in vitro} 3D cell invasion assay, such as the Matrigel sandwich (Srinivasan et al. 2009), could be optimised and employed for this purpose. It could be desirable to use the Matrigel sandwich assay in preference to the Transwell® assay, as preservation of the 3D culturing environment is essential to maintain the relevant morphological attributes and protein expression levels as reported here.

Further studies are also required to determine the link between AR and integrin \(\beta_1\). Considering the effect of 3D culture on the expression of AR protein in PC3 cells, it is
likely that integrin-ECM interactions may act to regulate AR. These studies could be performed using the integrin β1 inhibitory antibody, or using a shRNA/siRNA approach targeted to integrin β1, assessing the effect on AR protein expression, localisation and activity.

The consequences of the integrin β1-mediated interactions with chemokine receptors and stellate morphology in PC3 cells reported here allude to a central role for integrin β1. In order to understand this complex interaction, further studies are now needed to determine the functional impacts of integrin β1 regulation on CXCR7 and CXCR4. Firstly, the effects of integrin β1 inhibition observed here with inhibitory antibodies could be confirmed using an integrin β1 shRNA plasmid. Due to the length of time required for spheroid development to occur (6-7 days), a stable transfection approach could be suitable for investigations of transfected cells in 3D culture. Integrin β1 shRNA lentiviral plasmids are commercially available (Cat. No. sc-35674-SH; Santa Cruz Biotechnology), allowing for stable selection through the addition of puromycin. After transduction of PC3 cells with the lentiviral plasmid, and generation of a stable cell population, efficient knockdown of integrin β1 in this system could be confirmed via Western blot and immunocytochemistry. This system could then be used to investigate whether the loss of stellate morphology and down-regulation of CXCR7 and CXCR4 expression can be reproduced.

Next, the effect of integrin β1 knockdown and its regulation of cell phenotype could be investigated further. CXCR7 and CXCR4 are known to mediate cell adhesion, invasion and tumour angiogenesis (Chinni et al. 2006; Wang et al. 2008a). To determine the functional consequences of this regulation, PC3 cells stably expressing the integrin β1 shRNA could be employed in cell invasion assays towards the CXCR4/CXCR7 ligand, SDF-1α. Furthermore, the effect of integrin β1 inhibition on the SDF-1α-induced release of pro-angiogenic cytokines could be measured using commercially available ELISA kits (R&D Systems). Cell culture supernatants could be analysed for the expression of pro-angiogenic cytokines, including IL-8 and VEGF, which have been linked to SDF-1α-induced activities through CXCR7 and CXCR4 in PCa cells.

The regulation of tumour cell phenotype by integrin β1 could then be investigated using an in vivo approach. Specifically, these studies are required to determine whether tumour metastasis and angiogenesis are impacted by integrin β1 inhibition in vivo. PC3 cells stably expressing the integrin β1 shRNA could be implanted subcutaneously into 5-7 week old male SCID mice to generate PCa tumours (Wang et al. 2008a). After 5-6
weeks of growth, metastasis and invasion could be assessed (Wang et al. 2008a), and PCa tumour tissue sections could be obtained for immunohistochemical analysis of CXCR7 and CXCR4 protein expression. Tumour vascularisation could also be assessed, by staining tumour sections with an antibody for Factor VIII, which is expressed on blood vessels (Wang et al. 2008a). This could help to determine whether the observations of integrin β1 regulation of CXCR4 and CXCR7 in vitro correspond to altered tumour phenotype in vivo. In turn, these results could assist in more closely characterising the regulation of CXCR7 and CXCR4 by integrin-mediated interactions with the ECM. Indeed, integrins are under investigations for therapeutic inhibition in various types of cancer, with the aim of reducing metastasis (Bendas et al. 2012). In particular, an emerging anti-metastatic drug, low molecular weight heparin, has been demonstrated to act through integrin inhibition (Fritzsche et al. 2008). Interestingly, it has also been shown to bind to SDF-1α in vitro, potentially resulting in reduced tumour cell adhesion and migration via such modulation of chemokine network activity (Bendas et al. 2012). A deeper understanding of the regulation between integrins, chemokine receptors and tumour cell phenotype may result in the identification of therapeutic targets for inhibition of PCa metastasis.

7.4 Plasticity of PC3 Cell Morphology and Protein Expression in the Presence of Matrigel

The alterations of AR, CXCR4 and CXCR7 protein expression in 3D cultures of PC3 cells may not have been shared by LNCaP or DU145 cells due to the more mesenchymal phenotype of the PC3 cell line. Throughout these studies, the protein expression profile and phenotype of PC3 cells were more predisposed to manipulation through variations in culturing conditions when compared to DU145 or LNCaP cells. This may be due to an enhanced phenotypic plasticity in PC3 cells, perhaps conferred by the expression of mesenchymal markers in this cell line (Harma et al. 2010). Harma and colleagues (2010) reported a higher gene expression of mesenchymal markers (vimentin, fibronectin-1, N-cadherin, cadherin-2, cadherin-11) in PC3 cells, and loss of the epithelial marker E-cadherin, suggesting that these cells may have undergone an EMT transition to result in a more invasive, mesenchymal phenotype. In contrast, DU145 and LNCaP cells displayed more basal or luminal-associated epithelial expression profiles, respectively (Harma et al. 2010). The EMT process has been associated with enhanced tumour cell plasticity (Harma et al. 2010), and perhaps the
enhanced mesenchymal phenotype of PC3 cells may predispose their expression profile and phenotype to be influenced by the surrounding ECM.
Chapter Seven: Conclusions and Future Directions

7.5 Significance of Findings for PCa Research

Collectively, the results presented here can be combined to form a proposed model of positive feed-forward loop between cell-ECM interactions, CXCR7 and CXCR4 expression, and the AR in PCa cells (summarised in Fig. 7.1). Due to the established role of these receptors in the progression of PCa, the consequences of this positive feed-forward loop may contribute to the progression of PCa to local invasion and/or metastasis.

![Proposed mechanism for the regulation of protein expression by ECM interactions in PCa cells.](image)

The results presented in this thesis indicate that integrin-mediated ECM interactions can positively regulate CXCR4, CXCR7 and AR expression. Due to their established roles in PCa progression, this positive regulation network may contribute to progression of PCa.

For example, cells on the periphery of tumour cell masses may display enhanced integrin-mediated interactions with the ECM, to result in up-regulation of CXCR7 and CXCR4. Additionally, the effect of androgens on PCa cells results in a further up-regulation of CXCR4, which may be accompanied by a further enhancement of CXCR7 expression. Once expressed at high levels on the surface of tumour cells, these receptors could act to promote the migration and invasion of tumour cells towards their chemotactic ligands. Subsequently, local invasion and distant metastases are established, contributing towards the progression of PCa from local to advanced metastatic disease.

The model proposed here has implications for therapeutic approaches in the treatment of PCa. Firstly, subject to further investigation, CXCR7 may be a target for inhibition during early stage, androgen-sensitive PCa during the administration of ADT. Secondly,
depending on the results of further studies, the concurrent up-regulation of CXCR4 and CXCR7 protein observed here may suggest that a combined inhibition of both receptors may be required to effectively inhibit SDF-1α-induced activities in PCa progression. Thirdly, the influence of integrin β1 over the expression of CXCR7 and CXCR4 protein may suggest that the potential effectiveness of integrin inhibition in controlling metastasis can be directly linked with its regulation of these chemokine receptors.

A better understanding of these interactions in the presence of an ECM may contribute towards elucidating the regulatory mechanisms behind the progression of PCa to advanced disease. Importantly, future studies are required to trial combination therapies targeting multiple members of this network, with the aim to more effectively control the progression of PCa to advanced disease. Subsequent findings may lead to enhanced treatment options for patients with localised PCa that slow or halt the progression of disease to more advanced stages.
## Appendix A: Primers Used for DNA Sequencing Reactions

Table 8.1 Primers used for sequencing the AR gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Start*</th>
<th>Melting Temp (T\text{m}; °C)</th>
</tr>
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<tbody>
<tr>
<td>AR1</td>
<td>GATGAGGAACAGCAACCTT</td>
<td>331</td>
<td>49</td>
</tr>
<tr>
<td>AR2</td>
<td>CTGACCTTAAAGACATCCTGA</td>
<td>536</td>
<td>50</td>
</tr>
<tr>
<td>AR 870</td>
<td>CAAAGGTTTCTCTGCTAGAC</td>
<td>870</td>
<td>49</td>
</tr>
<tr>
<td>AR 1028</td>
<td>CTACCCCTGCTCTCTACA</td>
<td>1028</td>
<td>48</td>
</tr>
<tr>
<td>AR5</td>
<td>TGTTTTGCCATTTGACTATTACTTT</td>
<td>1638</td>
<td>51</td>
</tr>
<tr>
<td>AR6</td>
<td>AGCTGAAGAAAACCTGGTAATCT</td>
<td>1892</td>
<td>50</td>
</tr>
<tr>
<td>AR7</td>
<td>AACTGGGAGAGAGACAGCT</td>
<td>2120</td>
<td>51</td>
</tr>
<tr>
<td>T7</td>
<td>TAATACGACTCCTATAGGG</td>
<td>-</td>
<td>48</td>
</tr>
<tr>
<td>BGH</td>
<td>TAGAAGGCACAGTCTGAGG</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>M13 Forward (-20)</td>
<td>GTAAAACGACGGCCAG</td>
<td>-</td>
<td>46</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>CAGGAAACAGCTATGAC</td>
<td>-</td>
<td>45</td>
</tr>
</tbody>
</table>

*Number of bases into AR coding sequence
## APPENDIX B: PRIMER SEQUENCES FOR RT-PCR

Table 9.1 The sequences of primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Melting Temp (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 Forward</td>
<td>ATCTTCTGCCCACCATCTACTCCATCATC</td>
<td>63</td>
</tr>
<tr>
<td>CXCR4 Reverse</td>
<td>ATCCAGACGCAACATAGACCACCTTTTCA</td>
<td>61.6</td>
</tr>
<tr>
<td>AR Forward</td>
<td>GCCCGGAAGCTGAAGAAACT</td>
<td>53.8</td>
</tr>
<tr>
<td>AR Reverse</td>
<td>GAGTCGGGCTGGTTGTTGTC</td>
<td>55.9</td>
</tr>
<tr>
<td>PSA Forward</td>
<td>ACTGCATCAGGAACAAAGCGTGA</td>
<td>55.7</td>
</tr>
<tr>
<td>PSA Reverse</td>
<td>CGCACAACGTCAATGCGAAATAAC</td>
<td>55.7</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>GCCAAGGTTCATCCATGACAACCTTTGG</td>
<td>59.5</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>GCCTGCTTCACCACCTTTGATGTC</td>
<td>61.1</td>
</tr>
</tbody>
</table>
Chapter Seven: Conclusions and Future Directions

10 APPENDIX C: SEQUENCE OF THE AR INSERT IN PSG5AR
The sequence of the AR insert in PSG5-AR is shown below (5’-3’). The coding
region of the AR gene is underlined, accompanied by an adjacent 5’ and 3’ UTR
sequence. The presence of this sequence was verified in the PSG5-AR vector
using DNA sequencing analysis.
> readseq-43054_tmp_1 3433 bp
gcggagagaaccctctgttttcccccactctctctccacctcctcctgccttccccaccc
cgagtgcggagccagagatcaaaagatgaaaaggcagtcaggtcttcagtagccaaaaaa
caaaacaaacaaaaacaaaaaagccgaaataaaagaaaaagataataactcagttcttat
ttgcacctacttcagtggacactgaatttggaaggtggaggattttgtttttttctttta
agatctgggcatcttttgaatctacccttcaagtattaagagacagactgtgagcctagc
agggcagatcttgtccaccgtgtgtcttcttctgcacgagactttgaggctgtcagagcg
ctttttgcgtggttgctcccgcaagtttccttctctggagcttcccgcaggtgggcagct
agctgcagcgactaccgcatcatcacagcctgttgaactcttctgagcaagagaagggga
ggcggggtaagggaagtaggtggaagattcagccaagctcaaggatggaagtgcagttag
ggctgggaagggtctaccctcggccgccgtccaagacctaccgaggagctttccagaatc
tgttccagagcgtgcgcgaagtgatccagaacccgggccccaggcacccagaggccgcga
gcgcagcacctcccggcgccagtttgctgctgctgcagcagcagcagcagcagcagcagc
agcagcagcagcagcagcagcagcagcagcagcagcagcagcaagagactagccccaggc
agcagcagcagcagcagggtgaggatggttctccccaagcccatcgtagaggccccacag
gctacctggtcctggatgaggaacagcaaccttcacagccgcagtcggccctggagtgcc
accccgagagaggttgcgtcccagagcctggagccgccgtggccgccagcaaggggctgc
cgcagcagctgccagcacctccggacgaggatgactcagctgccccatccacgttgtccc
tgctgggccccactttccccggcttaagcagctgctccgctgaccttaaagacatcctga
gcgaggccagcaccatgcaactccttcagcaacagcagcaggaagcagtatccgaaggca
gcagcagcgggagagcgagggaggcctcgggggctcccacttcctccaaggacaattact
tagggggcacttcgaccatttctgacaacgccaaggagttgtgtaaggcagtgtcggtgt
ccatgggcctgggtgtggaggcgttggagcatctgagtccaggggaacagcttcgggggg
attgcatgtacgccccacttttgggagttccacccgctgtgcgtcccactccttgtgccc
cattggccgaatgcaaaggttctctgctagacgacagcgcaggcaagagcactgaagata
ctgctgagtattcccctttcaagggaggttacaccaaagggctagaaggcgagagcctag
gctgctctggcagcgctgcagcagggagctccgggacacttgaactgccgtctaccctgt
ctctctacaagtccggagcactggacgaggcagctgcgtaccagagtcgcgactactaca
actttccactggctctggccggaccgccgccccctccgccgcctccccatccccacgctc
gcatcaagctggagaacccgctggactacggcagcgcctgggcggctgcggcggcgcagt
gccgctatggggacctggcgagcctgcatggcgcgggtgcagcgggacccggttctgggt
caccctcagccgccgcttcctcatcctggcacactctcttcacagccgaagaaggccagt
tgtatggaccgtgtggtggtggtgggggtggtggcggcggcggcggcggcggcggcggcg
gcggcggcggcggcggcggcggcgaggcgggagctgtagccccctacggctacactcggc
cccctcaggggctggcgggccaggaaagcgacttcaccgcacctgatgtgtggtaccctg
gcggcatggtgagcagagtgccctatcccagtcccacttgtgtcaaaagcgaaatgggcc
cctggatggatagctactccggaccttacggggacatgcgtttggagactgccagggacc
atgttttgcccattgactattactttccaccccagaagacctgcctgatctgtggagatg
aagcttctgggtgtcactatggagctctcacatgtggaagctgcaaggtcttcttcaaaa
gagccgctgaagggaaacagaagtacctgtgcgccagcagaaatgattgcactattgata
aattccgaaggaaaaattgtccatcttgtcgtcttcggaaatgttatgaagcagggatga
ctctgggagcccggaagctgaagaaacttggtaatctgaaactacaggaggaaggagagg
cttccagcaccaccagccccactgaggagacaacccagaagctgacagtgtcacacattg
aaggctatgaatgtcagcccatctttctgaatgtcctggaagccattgagccaggtgtag
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tggtgtttgccatgggctggcgatccttcaccaatgtcaactccaggatgctctacttcg
cccctgatctggttttcaatgagtaccgcatgcacaagtcccggatgtacagccagtgtg
tccgaatgaggcacctctctcaagagtttggatggctccaaatcaccccccaggaattcc

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The open reading frame of AR insert (5’-3’) is listed below. Using DNA sequencing, the insert sequence within pENTR™-AR clone 2 was identified as the complete open reading frame of the AR gene using AR-specific primers listed in Methods section 2.7.8.1.
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