TMPRSS2 promoter driven enzyme-prodrug therapy for the treatment of PCa

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TMPRSS2 promoter driven enzyme-prodrug therapy for the treatment of PCa

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Declaration

I declare that the material presented in this thesis is the result of original work carried out by the author, Emma Jane Mercer, at the Centre for Molecular Oncology, Barts Cancer Institute, Barts and The London School of Medicine and Dentistry, Queen Mary, University of London. All external sources have been properly acknowledged.

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Abstract

Prostate cancer (PCa) progression is dependent on transcriptional activation of the androgen receptor (AR) in the majority of cases. Therefore, therapies include anti-androgens, which decrease levels of circulating androgens and inhibit AR activity. However, resistance inevitably develops, resulting in more aggressive, incurable late-stage disease with intact or constitutively active AR signalling. There is currently no cure for castration-resistant metastatic PCa and novel therapies are needed. In this work I aimed to develop a non-replicating adenovirus for delivery of a potent prodrug-converting enzyme expressed at high levels to specifically target and kill PCa cells.

TMPRSS2 is a major AR-regulated gene, commonly expressed at higher levels in cancer than normal prostate. In 40-70% of PCa cases the AR-regulated TMPRSS2 promoter is fused to oncogenic ERG. Based on this, we generated TMPRSS2 promoter and enhancer constructs, utilising promoter regions upstream of Exon1 and Exon2 of TMPRSS2 to drive the prodrug-converting chimeric enzyme cytosine deaminase uracil phosphoribosyl transferase (CD/UPRT) to investigate the therapeutic efficacy in various PCa models.

Prior work in the lab evaluated the selectivity and transcriptional efficiency of the various promoter and enhancer regions upstream of Exon1 and Exon2 in TMPRSS2 were investigated by Luciferase (Luc) expression. To further improve on promoter activity and transcription, the TMPRSS2 constructs were inserted into an expression cassette derived from the versatile expression vector VISA (VP16-GAL4-WPRE integrated systemic amplifier). When the Luc-gene was replaced with CD/UPRT in the VISA vector, specific and dose-dependent cell killing was observed in 22RV1 (AR+) cells when the non-toxic prodrug 5-flourocytosine (5-FC) was administered.

To increase transfection efficiency, I inserted the TMPRSS2-VISA-CD/UPRT expression cassette into a non-replicating adenovirus (Ad5-TV-CU) replacing the E1-genes. Ad5-TV-CU was characterized and protein expression of the CD/UPRT gene was detected at high levels post infection in AR-expressing 22RV1, LNCaP sublines, and VCaP cells, whilst remaining inactive in DU145 and HEK293 cells (AR-negative). Dose-dependent decreases in EC_{50}-values were observed upon

infection with Ad5-TV-CU in combination with low doses of 5-FC in a number of cell lines, including 22RV1, LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 cells, whilst demonstrating no cell killing at any concentration in PC3, PNT1a, PNT2 and PrEC cells (AR-).

To determine efficacy of the viral vector *in vivo*, I explored various AR-expressing PCa cell lines grown as xenografts in immunodeficient murine models, including 22RV1 and the LNCaP sublines LNCaP-104-S and LNCaP-CDXR3. However, establishment of the *in vivo* models was unsuccessful due to variable tumour take and growth (LNCaP) or tumours grew fast and studies had to be terminated prior to efficacy determination (22RV1).

Further comparison of the Exon1 TMPRSS2 promoter with the traditional PSA promoter and the chimeric PSA promoter/enhancer demonstrated the Exon1 TMPRSS2 construct to be superior by inducing 3.5-fold and 2.3-fold higher levels of luciferase expression than the PSA constructs, respectively. Investigation of both Exon1 and Exon2 TMPRSS2 promoters revealed that fusion of the two regions to form the chimeric Exon1/Exon2 promoter, drove higher levels of prostate specific expression, that have the potential to drive higher level expression than the SV40 promoter, this higher expression could improve efficacy of the current non-replicating Ad5-TV-CU.

In summary, Ad5-TV-CU has demonstrated efficacy in a number of AR expressing cell lines, with limited results *in vivo*. Further studies that incorporate the optimal LW chimeric promoter into a replicating adenoviral vector could dramatically improve the efficacy of this virus for future studies. Additionally the discovery of a more suitable *in vivo* model will help to establish the true therapeutic potential of the new optimal Ad5-TV-CU virus.

Abbreviations

5-FC: 5-flurocytosine

5-FU: 5-fluorouracil

Ad5: adenovirus type 5

Ad5-TV-CU- Ad5-TMPRSS2/VISA-CD/UPRT

ADP: adenovirus death protein

ADT: androgen deprivation therapy

AES: amino-terminus of split

AF1: activation function 1

AI: androgen independent

AR: androgen receptor

ARA: AR-associated

AREs: androgen-response elements

AS: active surveillance

ATCC: American Type Tissue Culture Collection

BCI: Barts Cancer Institute

BCa: breast cancer

Bica: Bicalutamide

bp: base-pair

BPH: Benign Prostate Hyperplasia

BSU: Biological services Unit

BSA: bovine serum albumin

CAB: combined androgen blockade

CAR: coxsackie adenovirus receptor

CBP: CREB-binding protein

CCKAR: cholecystokinin type A receptor

CD: cytosine deaminase

CDH8: chromodomain helicase DNA-binding protein 8

CD/UPRT: cytosine deaminase uracil phosphoribosyl transferase

cDNA complementary deoxyribonucleic acid

CgA: chromogranin A

CGH: comparative genomic hybridization

ChIP: chromatin immunoprecipitation

CKK: Cholecystokinin

CMV: cytomegalovirus

CO_2: carbon dioxide

CoRNR: corepressor nuclear receptor

CPA: cyproterone acetate

CPE: cytopathic effect

CR: conserved region

CRPC: castration resistant PCa

CsCl: caesium chloride

CTD: c-terminal domain

Ctl: control

CYP: Cytochrome P450

DBD: DNA-binding domain

DBP: DNA-binding protein

DHEA: dehydroepiandrosterone

DHT: dihydrotestosterone

DJBP: DJ1-binding protein

DMEM: Dulbecco's Modified Eagle medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DNTP: deoxyribonucleotide triphosphate

DRE: digital rectal exam

DTT: dithiothreitol

EBP1: ErbB3-binding protein

EC50: effective concentration dose required to kill 50% of cells

ECL: Enhanced Chemiluminescence

EEV: extracellular enveloped viruses

EGF: epidermal growth factor

EMT: epithelial mesenchymal transition

Enz: enzalutamide

ER: Oestrogen receptor

FACS: florescent activated cell sorting

FCS: fetal calf serum

FDA: Federal Drug Administration

FSH: follicle stimulating hormone

FSC: forward side scatter

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

gDNA: genomic DNA
GFP: green fluorescence protein
GCV: ganciclovir
GDEPT: Gene Directed Enzyme Prodrug Therapy
GJIC: Gap Junctional Intercellular Communication
GM-CSF: granulocyte macrophage colony-stimulating factor
GMP: good manufacturing practice
GnRH: Gonadotropin-releasing hormone
GWAS: genome wide association study
h: hours
H&E: hematoxylin and eosin
H_2O: water
HAT: histone acetyltransferase
HDAC: histone deacetylases
HGPIN: high-grade prostatic intraepithelial neoplasia
hK2: human glandular kallikrein 2
HiR: hinge region
HR: homologous recombination
HRPC: hormone refractory PCa
HSP: heat shock protein
HSPG: heparin sulphate proteoglycans
HSV: herpes simplex virus
HSV-tk: herpes simplex virus thymidine kinase
IGF-1: insulin-like growth factor 1
IHC: immunohistochemistry
IL: interleukin
IMRT: intensity modulated radiotherapy
IP: intraperitoneal
ISGs: interferon stimulated genes
It: intratumoural
ITR: inverted terminal repeat
kDa: kiloDalton
lats: large tumour suppressor 2
LB: lysogeny broth bacteria media
LBD: ligand-binding domain
LH: luteinizing hormone

LHRH: luteinizing hormone-releasing hormone
MAPK: mitogen-activated protein kinase
Mb: Megabase
Mab: monoclonal antibody
MHC: major histocompatibility complex
min: minutes
mRNA: messenger RNA
miRNA: micro RNA
MLP: major late promoter
MOI: multiplicity of infection
MTS: 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
n: number of times an experiment was repeated
NEB: new England biolabs
NCoR: nuclear receptor corepressor
NF1: nuclear factor 1
ns: not significant
NTD: N-terminal domain
NTR nitroreductase
OC: osteocalcin
OD: optical density
OriR : Origin of Replication
PAK6: p21-activated kinase
PBS: phosphate buffered saline
PCa: PCa
PCR: polymerase chain reaction
pfu: plaque forming unit
PIA: prostatic inflammatory atrophy
PISA: protein inhibitor of activated STAT
PIC: preinitiation complex
PIN: prostatic intraepithelial neoplasia
PMS: phenazine methosulphate
ppc: particles per cell
pRB: retinoblastoma tumour suppressor protein
PolyQ: polyglutamine
PSA: prostate specific antigen

PSMA: Prostate specific membrane antigen

PTEN: phosphatase and tensin homologue deleted on chromosome 10

PVDF: polyvinylidene difluoride

PYK2: proline-rich tyrosine kinase 2

rpm: revolutions per minute

QPCR: quantitative polymerase chain reaction

RGD: arginine-glycine-aspartic acid

RLU: raw light units

RNA: ribonucleic acid

RPMI: Roswell Park Memorial Institute medium

RSV: rous sarcoma virus promoter

RT: room temperature

SCID-X1: X-linked severe combined immunodeficiency

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: standard error of the mean

SMRT: silencing mediator of retinoic acid

SNP: single nucleotide polymorphism

SRC: steroid-receptor-coactivator

SSC: right angle side scatter

STR: short tandem repeat

TARP: T-cell receptor gamma-chain alternate reading frame

TAU: transactivation domain

TBE- Tris Borate EDTA

TBP: TATA-binding protein

TCID50: tissue culture infectious dose 50%

TGF-β: transforming growth factor-β

TGIF: TG-interacting factor

TK: thymidine kinase

TMPRSS2: transmembrane protease serine 2

TNM: tissue lymph node metastasis

TNS: trypsin neutralising solution

TP: terminal protein

TRAMP: transgenic adenocarcinoma of the mouse prostate

TRUS: transurectal ultrasound

TS: thymidylate synthase

TSG: tumour suppressor gene

TSP: tissue specific promoter

TSS: transcription start site

TSTA: Two Step Transcriptional Amplification

UV: ultra violet

VA: virus associated

VCAM: vascular adhesion molecule

VDR: vitamin D receptor

vp: virus particle

wt: Wild type

XMRV: xenotropic murine leukemia virus-related virus

ºC: Degrees Celsius

µg: microgram

L: litre

mL: millilitre

µL: microlitre

M: Molar

mM: millimolar

µM: micromolar

nM: nanomolar

mg: milligram

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CHAPTER 1

INTRODUCTION

1.1 The normal prostate, PCa aetiology and PCa diagnosis

1.1.1 Anatomy of the Prostate.

The prostate is the size of a walnut and is located around the neck of the bladder and urethra, just in front of the rectum in men (Shen and Abate-Shen 2010). During fetal development, at around 10 weeks, the budding of the prostate epithelium is stimulated from the urogenital sinus, composed of urogenital sinus epithelium and urogenital sinus mesenchyme, by fetal androgens that are essential for normal prostate development (Cronauer et al. 2003). As a bi-product of this action, growth factors are released to activate the mesenchyme cells which express high levels of androgen receptor (AR). The development of the normal prostate is dependent on the interaction between the urogenital sinus epithelial cells and the urogenital sinus mesenchyme cells. Expression of AR from the mesenchyme cells results in induction of epithelial bud formation, bud growth and ductal branching, promotion of epithelial differentiation into secretory epithelial cells, which are AR-positive, and release of paracrine growth factors from the mesenchyme cells that stimulate glandular morphogenesis and epithelial cell growth (Cunha et al. 2003). As a reciprocal result of epithelial cell differentiation, the developing prostatic epithelium induces smooth muscle differentiation in the urogenital sinus mesenchyme, resulting in development of the mesenchyme into a mature prostatic stroma, consisting mainly of smooth muscle cells and the epithelium displaying a highly differentiated phenotype.

The adult prostate is partly muscular, partly glandular, composing of a glandular epithelial and a fibromuscular stroma compartment. It has a zonal architecture which includes central and large peripheral zones contained within the glandular portion of the prostate, constituting 95% of the gland. The remaining 5% is composed of the transition zone and periurethral glands that share a similar acinar structure due to their common origin, the urogenital sinus (Figure 1). In contrast, the glands of the central zone, both originating from the Wolffian duct are morphologically distinct.

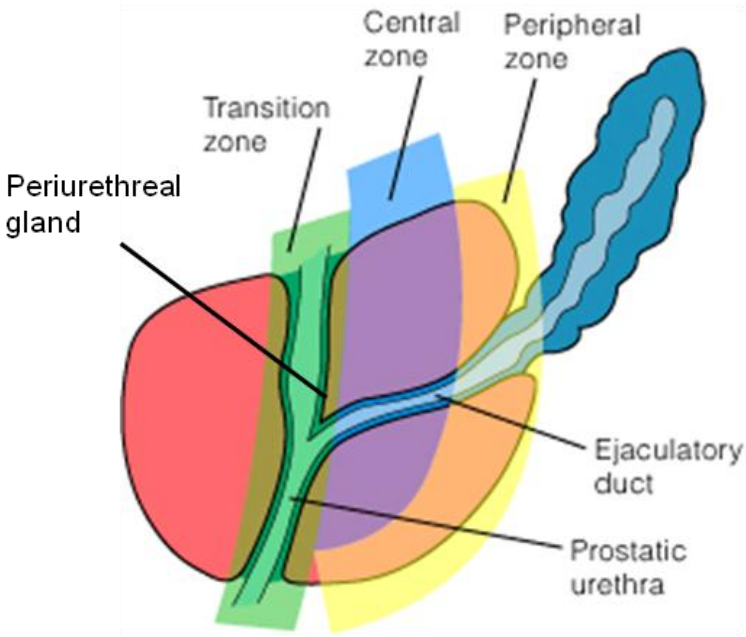


Figure 1 Two-dimensional schematic showing the zonal architecture of the prostate

95% of the prostate is composed of the central and peripheral zones, with the remaining 5 % consisting of the transitional zone and the periurethral gland region.

The outermost peripheral zone harbours the majority of prostate carcinomas (about 60-70%) and occupies the largest volume (Shen and Abate-Shen 2010, Cronauer et al. 2003). The primary function of the prostate is to secrete a slightly alkaline fluid (~pH 7.2-8) that constitutes the majority of the seminal fluid, the fluid that carries sperm and protects it from the acidic environment of the vagina, as well as protecting the male urinary and reproductive system from pathogens. There is a medium-high prevalence of cancers that occur in the peripheral gland, fewer in the transition zone and a very low number in the central zone. Large areas of inflammation can be found around the transition zone, where most benign prostate hyperplasia (BPH) cases develop, most often in combination with focal atrophy. Acute inflammation is also apparent in the transition zone as well as the peripheral zone. Most cases of high-grade prostatic intraepithelial neoplasia (HGPIN) occur in both the peripheral and transition zones as seen with Carcinomas (Table 1)

Table 1 Table showing the prevelance of PCa in the different areas of the prostate.

Zone/ condition	Focal atrophy	Acute inflammation	Chronic inflammation	BPH	HGPIN	Carcinoma
Peripheral	✓✓✓	✓	✓✓		✓✓	✓✓
Transition	✓✓	✓	✓✓	✓✓✓	✓	✓
Central	✓		✓	✓	✓	

✓✓✓= high prevalence, ✓✓=medium-high prevalence, ✓= low prevalence. Blank boxes = no cases. BPH= benign prostate hyperplasia, HGPIN= high grade prostatic intraepithelial neoplasia.

The human prostate contains a pseudostratified epithelium consisting of three differentiated epithelial cell types: Luminal, basal and neuroendocrine cells (Shen and Abate-Shen 2010) (Figure 2). The luminal cells produce protein secretions and express cytokeratins 8 and 18 among other markers including high levels of AR. The basal cells are located below the luminal epithelium and express AR at very low levels along with p63 and cytokeratins 5 and 14. The neuroendocrine cells are morphologically undistinguishable from basal cells and are thought to regulate the growth, differentiation and secretory function of the prostate gland (Abate-Shen and Shen 2000a, Sun, Niu, and Huang 2009). Neuroendocrine cells are very rare and express no AR but do express chromogranin A and synaptophysin (Shen and Abate-Shen 2010).

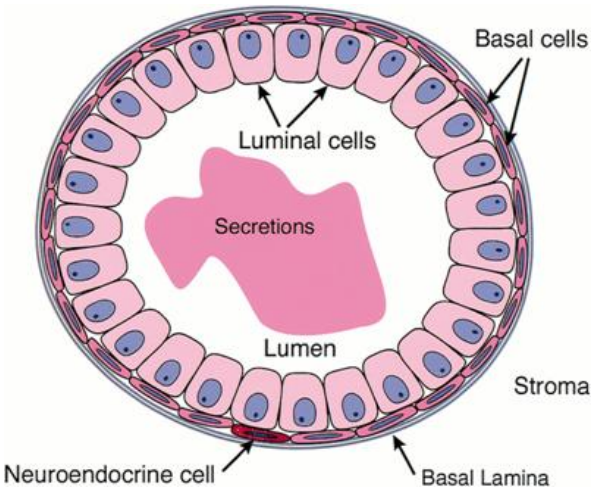


Figure 2 Schematic illustration of the different cell types in the human prostatic duct

The human prostate comprises three differentiated epithelial cell types, luminal, basal and endocrine. These cell types differ in their protein secretions, each of which has a different function in driving prostate cell growth (Abate-Shen and Shen 2000).

1.1.2 Aetiology of PCa

In 1863 Virchow proposed a causal link between chronic inflammation and carcinogenesis that is now widely accepted (Balkwill and Mantovani 2001). It is estimated that 25% of all malignancies are connected to chronic inflammation (Woenckhaus and Fenic 2008). This has been confirmed in patients who suffer from Crohn's disease or ulcerative colitis, who have a 10-fold increased risk of developing colon cancer (Itzkowitz and Yio 2004, Seril et al. 2003). Similarly, inflammation of the prostate has been proposed as a potential precursor to prostate cancer (PCa).

Regions of prostatic inflammatory atrophy (PIA) are associated with inflammatory cell infiltrates and develop at high frequencies to include large regions of the prostate in some men (De Marzo et al. 1999). This often includes a reduction in the volume of pre-existing glands and stroma (McNeal 1988). This process occurs in response to unknown stimuli and features atrophic epithelial cells that regenerate in response to cellular damage. These cells have been shown to merge with high grade prostatic intraepithelial neoplasia (HGPIN) and contain some of the well recognised genetic mutations that are found in PCa (De Marzo et al. 2007). PIA has been found in 30% of prostate biopsies, has a lower association to PCa than HGPIN and is associated with low grade tumours (Celma et al. 2014). Despite evidence suggestive of a role for PIA in prostate carcinogenesis, the mechanisms that would permit its neoplastic transformation are still unknown. However, evidence suggests a model in which PIA may progress into prostate intraepithelial neoplasia (PIN) and/or invasive PCa (Putzi and De Marzo 2000). Further evidence needs to be gathered to fully elucidate the role of PIA in the development of PCa.

PIN is widely believed to be the precursor of PCa (Figure 3), although this is yet to be conclusively proven (Shen and Abate-Shen 2010). PIN is described as the abnormal proliferation of the secretory epithelium within prostatic ducts and acini, without the invasion of the basement membrane (Godoy and Taneja 2008). HGPIN has been recognised as a risk factor for PCa and is found in 85-100% of radical prostatectomy specimens, and tissue adherent to PCa tumours (Godoy and Taneja 2008). Characteristics typical of PIN include appearance of luminal epithelial hyperplasia, reduction in basal cells, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia, and nuclear atypia (Godoy and Taneja 2008). PIN

displays a reduced number of basal cells, which are entirely absent in PCa. The difference between HGPIN and PIN is defined by the presence of prominent nucleoli. HGPIN can be identified due to an increase in cellular proliferation markers and the incidence and severity of HGPIN has been shown to increase with age, similar to the development of PCa (Qian, Wollan, and Bostwick 1997). HGPIN also has similar patterns of prevalence to PCa, with increased incidence in African- American men compared with Caucasian men (Sakr et al. 1995).

PCa is frequently multifocal, resulting in huge heterogeneity. As a result of this cellular dysplasia, tissue disorganization and genetic alterations vary massively within these distinct foci. It is widely accepted that PCa can arise from multiple independent foci and is therefore polyclonal in origin. However, some studies have suggested a monoclonal origin for PCa, as identical genomic copy number changes were detected through analysis of genome-wide DNA copy number, in a high resolution SNP array study, in isolated individual cancer and precursor legions (Boyd, Mao, and Lu 2012). This study also recorded extensive subclonal genomic alterations for all tumours, suggestive of interfocal heterogeneity.

PCas are largely indolent with respect to growth and only one third become locally invasive, spreading beyond the tissue capsule or metastasizing to local lymph nodes and distal organs, including bone, liver and lung (Schulz, Burchardt, and Cronauer 2003). In contrast to the normal prostate, the luminal cells express high levels of AR, resulting in enhanced cell growth in the early stages of PCa development. Consequently, androgen withdrawal (androgen deprivation therapy, ADT) is the mainstay therapy for the treatment of PCa, but will only induce apoptosis in androgen-dependent cells. Due to the heterogeneous nature of PCa (Bastus et al. 2010), where not all cells are reliant on androgen stimulation for growth, ADT will select for those cells that are androgen-independent, leading to the occurrence of a castration resistant PCa phenotype (discussed later in more detail) (Litvinov, De Marzo, and Isaacs 2003).

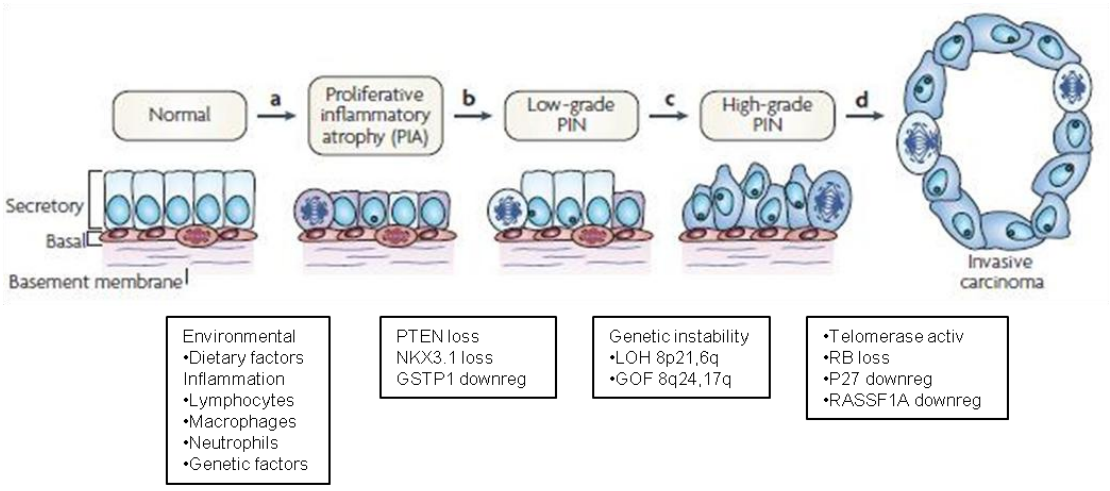


Figure 3 Progression pathway to PCa

The progression from normal prostate tissue to metastasis. Normal cells progress to atrophy or proliferative inflammatory atrophy (PIA). From this stage they progress to prostatic intraepithelial neoplasia (PIN) and then on to invasive carcinoma and, eventually, metastasis. Adapted from (De Marzo et al. 2007).

1.1.3 PCa Statistics.

PCa is the second biggest cause of cancer-related death in men in the western world, accounting for 12% of deaths (statistics 2014). In the UK, 37,000 men are diagnosed with PCa every year and 9,698 male PCa deaths were recorded in England and Wales in 2012, accounting for 4% of total deaths (Statistics 2012). Estimates suggest that the incidence of PCa is set to rise to >61,000 cases per year by 2030 (Mistry et al. 2011). PCa is the second most frequently diagnosed cancer worldwide and the sixth leading cause of cancer death, accounting for 6% of total cancer deaths in 2008 (Jemal et al. 2011). Incidence rates of PCa vary by more than 25-fold worldwide (Jemal et al. 2011). More developed countries have a higher incidence of the disease, thought to be due to a number of reasons including the frequency of Prostate Specific Antigen (PSA) testing, diet, lifestyle and genetic background.

PCa is a slow growing, progressive disease and as a result has become known as a ‘disease of the elderly’. Autopsy studies showed that a large number of men die of different illnesses before the cancer is detected and 73% of PCa deaths occur in men over the age of 75. Despite the lack of a cure for PCa, the 5-year survival rates post diagnosis are still at 81.4% (http://www.ons.gov.uk/ons/rel/vsob1/mortality-statistics--deaths-registered-in-england-and-wales--series-dr-/2012/stb-deaths-registered-in-england-and-wales-

in-2012-by-cause.html). This is most probably due to the detection of early stage non-life threatening PCa by regular testing for PSA. The majority of PCas are indolent, unlike many other cancers, which even when detected at an early stage will kill without an effective treatment, discussed further in section 1.1.4.1.

1.1.4 PCa diagnosis, grading and staging

Traditionally, detection of PCa relied on a digital rectal examination (DRE) (Damber and Aus). Approximately 18% of PCa cases were detected by a PCa suggestive finding via DRE alone, regardless of PSA level (Carvalhal et al. 1999). However, over recent years the application of PSA serum concentration as a biomarker and transrectal ultrasound (TRUS)- guided biopsy have revolutionised the diagnosis and treatment of PCa, facilitating screening programs to diagnose larger numbers of men at earlier stages of the disease (Nash and Melezinek 2000). This allows for disease detection when the cancer is indolent and would otherwise be left undetected, most probably accounting for the increase in survival rates due to the combination of earlier treatment and the detection of many indolent cancers that would never have killed the patients, even without treatment.

1.1.4.1 Prostate Specific Antigen (PSA)

PSA was first investigated as a serum biomarker for adenocarcinoma of the prostate in 1987 (Stamey et al. 1987). It has since become standard practice to measure serum testosterone levels in men over the age of 50 if they present with symptoms of PCa, but there is currently no organised screening program in the UK for PCa, and screening differs between countries. Until recently, yearly PSA screening was recommended in the United States for men over 50, however some advisory groups are now advising against regular testing due to the potential harms outweighing benefits, although yearly testing is still carried out through Medicare insurance.

PSA is specific to the prostate as it is produced in normal prostate secretions. Increases in the levels of PSA are a hallmark of prostate cancer, however raised PSA levels do not necessarily indicate cancer. High PSA levels can also be due to benign prostatic hypertrophy (BPH), prostatitis or urinary infection (Nadler et al. 1995). Normal PSA levels are 0-4 ng/ml for men aged up to 70 years and can rise to 5 ng/ml for men aged over 70 (Nash and Melezinek 2000). The age specific cut-offs in normal PSA measurement recommended by the PCa risk management

program in the UK are: 50-59 years ≥ 3 ng/ml: 60-69 years ≥ 4 ng/ml: 70 years and above ≥ 5 ng/ml. Studies have shown that approximately 80% of patients with PCa and 20% of patients with BPH have PSA levels exceeding 4 ng/ml (Catalona, Richie, Ahmann, Hudson, et al. 1994). Thus, elevated levels of PSA antigen in the blood indicate that further investigation is necessary to identify the cause. Conflicting with this evidence, 2% of men that present with fast growing PCa have a normal PSA level, suggesting that PSA is not a totally reliable biomarker for the detection of PCa. Men who present with high PSA levels will have a biopsy to confirm if cancer is present and approximately 25% of biopsy cases return with a positive diagnosis (Smith, Humphrey, and Catalona 1997). In order to establish the stage and grade of the cancer, the patient may be given a 'TNM' assessment (although this is not currently as popular) and a Gleason score (described more fully below). This score will determine the intensity of any resulting treatment.

1.1.4.2 Gleason score.

The Gleason score has been established as being one of the most informative grading systems to determine the stage and prognosis for PCa. It was developed in 1974 by Dr Donald Gleason, who based the score on biopsies from almost 3,000 patients with PCa (Gleason and Mellinger 1974)**,** and it is currently used to grade PCa based on core biopsies. It is calculated by the addition of the two most common patterns (grades 1-5) of tumour that are found and so ranges between 2 and 10. However, more recently, individual Gleason scores of 1 or 2 are not defined as cancer, therefore a combined score of 6 is regarded as showing the least aggressive form of disease and a score of 10 showing the most aggressive (see Table 2) (Gleason and Mellinger 1974).

Table 2 Gleason score

Gleason score	What does it mean?
3+3	All cancer cells proliferate slowly
3+4	Most cancer cells proliferate slowly, however some cells grow at a moderate rate
4+3	Most cancer cells proliferate at a moderate rate, but some proliferate more slowly
4+4	All cancer cells proliferate at a moderate rate
4+5	Most cancer cells proliferate at a moderate rate, however some cells rapidly proliferate
5+4	Most cancer cells proliferate quickly
5+5	All cancer cells proliferate quickly

1.1.4.3 Tumour, lymph node, metastasis (TNM) classification of malignant tumours

PCa is also staged using the TNM system (Figure 4, Table 3), which separately assesses tumour (T), lymph nodes (N) and secondary cancer (metastases-M). Together with PSA levels and Gleason score to categorize PCa into 5 stages (Sobin, Gospodarowicz, and Wittekind 2010) **(**Figure 4). TNM was devised by Pierre Denoix between 1943 and 1952 and is a globally recognised standard for classifying the extent and spread of cancer.

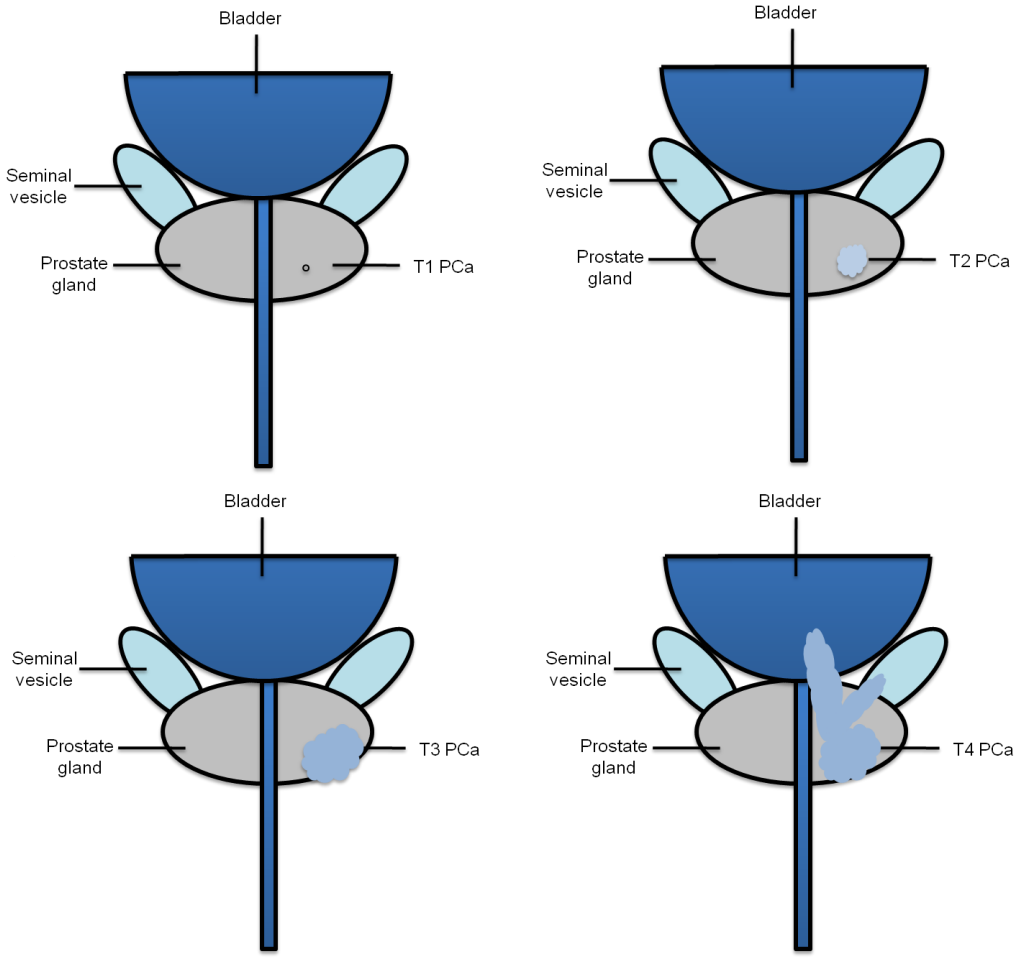


Figure 4 Diagrammatic illustrating tumour grades 1-4

T1- localised to the prostate. T2 tumours are restricted to the prostate but occupy a larger area of the prostate that can extend to both lobes. T3 tumours are locally advanced and have spread outside the prostate but not yet invaded other organs. T4 tumours have spread to nearby organs such as the bladder.

T (tumour staging) is subdivided into T1-T4. T1 tumours are most often discovered by needle biopsy that has been performed as a result of raised PSA levels. T1

stage tumours cannot be seen on scans or felt during examination. T2 tumours are restricted to the prostate gland and can be subdivided into 3 smaller groups; T2a- present in only half of one of the prostate gland lobes, T2b- present in more than half of one of the lobes and T2c- present in both lobes but still restricted to the prostate gland. T3 tumours are locally advanced cancers that have spread outside the prostate gland, but have not yet infiltrated other organs. T3 tumours are subdivided into 2 further groups; T3a- the tumour has broken through the prostate gland and T3b- the tumour has spread into the seminal vesicles. T4 tumours are also locally advanced, but have spread into nearby organs, such as the bladder.

N (lymph node) staging- Metastatic PCa can spread to the lymph nodes, causing inflammation. Lymph node staging can be divided into 3 groups; NX- lymph nodes cannot be checked, N0- no cancer cells can be detected in lymph nodes close to the prostate or N1- there are cancer cells present in lymph nodes.

Table 3 PCa overall staging

Stage	T score	N score	M score	Gleason score	PSA levels (ng/ml)
I	1	0	0	≤6	<10
	2a	0	0	≤6	<10
IIA	1	0	0	<7	<20
	1	0	0	≤6	≥10<20
	2a/2b	0	0	≤7	<20
IIB	2c	0	0	Any	Any
	1/2	0	0	Any	≥20
	1/2	0	0	≥8	Any
III	3	0	0	Any	Any
IV	4	0	0	Any	Any
	Any	1	0	Any	Any
	Any	Any	1	Any	Any

M staging- metastases (Metastatic cancer is cancer that has spread to other parts of the body, most commonly the bone and lymph nodes), sub-categorized into 5 groups (M0, M1, M1a, M1b and M1c). M0- cancer has not spread outside the pelvis, M1- cancer has spread outside the pelvis, M1a- cancer cells can be found in the lymph nodes outside the pelvis, M1b, cancer cells can be found in the bone and finally M1c, cancer cells can be found in other parts of the body.

1.2 Genomic alterations in PCa.

Cancer is recognised as a genetic disease and PCa is both very heterogeneous and characterised by somatic copy number alterations, point mutations and structural rearrangements that cause loss of function of tumour suppressor genes (TSGs) or overexpression of oncogenes. Genetic alterations can be inherited (germline mutations), or induced by endogenous or exogenous carcinogenic factors (somatic alterations). Epigenetic modifications to the DNA including DNA methylation and histone accetylation have also been shown to contribute towards prostate carcinogenesis. As well as pathological heterogeneity, PCa is also highly heterogeneous when presented clinically. Many same stage tumours can lead to different clinical outcomes ranging from latent to highly aggressive disease states despite tumours being considered as histologically identical (Boyd, Mao, and Lu 2012).

1.2.1 Hereditary PCa

A familial history of PCa is an important risk factor in PCa development. Individuals who have a first degree relative with a history of PCa have a two-fold risk of developing cancer compared with the general population (Carter et al. 1992, Edwards and Eeles 2004). In some cases hereditary cancers are a result of an inherited mutation in one allele of a tumour suppressor gene, requiring one further somatic mutation in the second allele for the development of cancer (Schulz, Burchardt, and Cronauer 2003). 40% of hereditary PCa is diagnosed by the age of 55 and accounts for 9% of total PCas (Carter et al. 1992). Due to the age at which hereditary cancer presents, genotype data are often unavailable for older relatives of the patient. Despite this, several regions of the genome have been identified as containing PCa predisposition genes through linkage studies, including; 1q24-1q25 (HPC1) containing the RNASEL gene which is susceptible to both nonsense and frameshift mutations (Xu 2000), 1q42-q43 (PCAP) (Berthon et al. 1998), 8p22-23 (PANX1) (Xu et al. 2001), Xq27-q28 (HPCX), 1p36 (CAPB) (Gibbs et al. 1999), 20q13 (HPC20), 17p11 (ELAC2) that suffers nonsense or missense mutations (Tavtigian et al. 2001) and 16q23 (Nwosu et al. 2001, Bratt 2002, Simard et al. 2002). The results of several linkage studies were later reanalysed as a combined dataset. This new dataset represented 426 families

with hereditary PCa and allowed for identification of a new susceptibility loci at 17q21-22 (BRCA1) (Gillanders et al. 2004).

In 2006 the application of a new analysis technology, genome wide association studies (GWAS), identified a 3.8Mb region on the 8q24 chromosome band, containing the MYC oncogene (Amundadottir et al. 2006). Subsequently, 29 SNP loci on various chromosomes have been identified in addition to 9 SNP loci on 8q24. The latter of which were shown to be independently associated with PCa risk. A later study identified a further seven susceptibility loci (Boyd, Mao, and Lu 2012, Kote-Jarai et al. 2011).

Germline mutations in the AR are rarely identified in patients with PCa. However, prevalent polymorphisms have also been described as contributing towards the genetic susceptibility of PCa. Polymorphisms in the steroid hormone receptor AR, include differences in CAG and GGN repeats encoding glutamine and glycine repeats respectively, in the N terminal transcriptional activation domain of AR. Studies have suggested that shorter CAG repeats are associated with a small increased risk of PCa (Wang et al. 2014), although the true association between CAG and GGN repeat length and PCa risk remains unclear (Zeegers et al. 2004, Gu et al. 2012). A population disparity exists between CAG repeat lengths in white men, Asian men and black men. CAG repeat length is shortest in black men and longest in Asian men, with white men in the middle (Hsing et al. 2000). Interestingly this disparity also correlates with ethnicity based variation in PCa incidence and mortality (Buchanan et al. 2001). Despite extensive investigation into the association of CAG repeat length and PCa incidence a true causal link has not yet been conclusively proven.

Polymorphisms in genes associate with androgen biosynthesis are also frequently observed. The SRD5A2 gene contains a V89L polymorphism associated with decreased DHT production *in vitro* and in *vivo* (Hsing et al. 2001). SRD5A2 encodes part of 5-α-reductase and the V89L polymorphism is more common in Chinese and Japanese men, contradicting PCa ethnical disparity studies in which PCa is less prevalent in the Asian population. A number of other common polymorphisms in the androgen biosynthesis pathway occur in CYP17A1, a gene which encodes the cytochrome P450 (CYP) subfamily 17A enzyme. This enzyme mediates 17 α-hydroxylase and 17, 20 lyase activities. Other genes required for androgen biosynthesis and susceptible to polymorphisms include; CYP19A,

CYP3A HSD3B and HSD17B (Mononen and Schleutker 2009, Li et al. 2013). Polymorphisms have also been detected in the vitamin D receptor (VDR), where different lengths of a polyA-repeat in the 3' region confer susceptibility to PCa (Ingles et al. 1997).

1.2.2 Somatic DNA alterations in PCa

Somatic copy number alterations are present in 90% of primary prostate tumours and more commonly occur as deletions rather than amplifications (Schoenborn, Nelson, and Fang 2013). In localised cancer these somatic alterations occur in only a small portion of the genome, progressing to large proportions of the genome in metastatic disease, suggesting increased genomic instability. Several gains and losses of chromosomal regions have been identified by comparative genomic hybridization (CGH), including gain at 8q and losses at 3p, 8p, 10q, 13q, and 17p (Dong 2001, Lapointe, Li, et al. 2007). Frequent deletions of 8p, 10q and 13q encompass genes such as NKX3-1, PTEN, BRCA2 and RB1. Frequent amplifications are seen in chromosomes X, 7, 8q and 9q in Castration resistant metastatic tumours and include AR at Xq12 and MYC oncogenes at 8q24.

Unlike copy number alterations, point mutations are uncommon in PCa and result in both missense and nonsense mutations (Barbieri et al. 2013). Point mutations are more common in CRPC cases that harbour genomic alterations of the AR, occurring in 20% of cases (Beltran et al. 2013). Primary PCa has a somatic mutation rate of $1\text{-}2x10^{-6}$ (Schoenborn, Nelson, and Fang 2013). Of the thousands of mutations that can exist within one tumour only ~20 are likely to impact on protein stability and function (Schoenborn, Nelson, and Fang 2013). However, mutations in essential genes such as MSH6, a DNA mismatch repair enzyme, have been associated with hypermutator phenotypes. These hypermutators result in 25-fold more mutations than are seen in normal PCa (Taylor et al. 2010, Kumar et al. 2011). Point mutations also occur in the tumour suppressor genes TP53, PTEN, RB1 and the PIK3CA oncogene in a very small proportion of PCas, as well as mutations in other genes including KRAS and BRAF, resulting in an oncogenic phenotype.

Other genetic alterations in cancer include chromosomal rearrangements, caused by double strand breaks that occur during replication or transcription as a consequence of DNA unwinding. Inefficient repair of these breaks can result in intra and inter chromosome rearrangement, sometimes resulting in the production

of fusion genes (Schoenborn, Nelson, and Fang 2013). Understanding how these genes contribute to cancer is important, as they can act at the molecular level, either by driving the overexpression of an oncogene, or by the production of a new fusion protein that promotes oncogenic activity. One of the most commonly recognized genomic alterations is the *BCR:ABL* fusion gene, otherwise known as the Philadelphia chromosome in Chronic Myeloid Leukaemia (Sawyers 1999). With the identification of the novel tyrosine kinase inhibitor, imatinib, successful treatment for this cancer has since become available (Druker et al. 2001). One of the most frequently observed alterations in human cancer is the fusion of the AR-driven *TMPRSS2* upstream region with the *ERG* gene (Tomlins et al. 2005) (see section below).

1.2.3 ETS fusion genes in cancer

The ETS fusions genes were first discovered when strong outlier profiles of two ETS family genes, *ERG* and *ETV1* were identified by oncomine. After this initial discovery RLM-RACE studies and subsequent sequencing anaylsis revealed fusion of *ERG* and *ETV1* with the androgen responsive promoter region of TMPRSS2, a transmembrane serine protease (Tomlins et al. 2005). Fusions between TMPRSS2 and other ETS variants including *ETV4*, *ETV5* and *ELK4* were subsequently identified but are not as prevalent as the *TMPRSS2:ERG* fusion (Tomlins et al. 2006, Helgeson et al. 2008). As well as *TMPRSS2* a number of alternative 5' fusion partners have been identified in ETS gene fusions, including SLC45A3, ERVK-24, HNRPA2B1, C150RF21 and NDRG1 (Lapointe, Kim, et al. 2007, Pflueger et al. 2009), of which SLC453A and NDRG1 are also androgen responsive, suggesting that androgen responsive genes are prone to creating recurrent gene fusions or have a selective cell growth advantage in PCa.

1.2.3.1 *TMPRSS2:ERG* fusion.

TMPRSS2 is a transmembrane serine protease and belongs to the serine protease family. Serine proteases are known to be involved in many physiological and pathological processes (http://www.genecards.org/). *TMPRSS2* promoter expression has been demonstrated to be prostate specific, with the promoter activated by binding of AR to androgen response element(s) (AREs) in its 5'-end. However, low levels of expression have also been found in the colon, liver, lung, kidney and pancreas (Lin et al. 1999). Studies have shown that the levels of *TMPRSS2* expression are higher in PCa tissues in comparison to benign prostate

tissue (Vaarala et al. 2001). The *TMPRSS2*:*ERG* fusion gene accounts for 40-70% of all reported ETS fusion genes and is found in more than 50% of PCa patients (Tomlins et al. 2005).

The role of *TMPRSS2:ERG* fusion is not fully elucidated. It is possible that the fusion gene may play a role in disease progression through increased levels of *ERG* expression, and could therefore possibly be used as a marker to differentiate between localised cancer, and more advanced aggressive forms of the disease. Overexpression of *ERG* has been shown to promote the invasive potential of PCa cells (Li et al. 2011). Patients with *TMPRSS2:ERG* fusion gene have shown a higher risk of disease recurrence (58.4% at 5 years) in comparison to *TMPRSS2:ERG* negative patients (8% recurrence) (Nam et al. 2007). However, similar studies have shown opposing outcomes (FitzGerald et al. 2008, Gopalan et al. 2009, Fine et al. 2010). Additionally, *TMPRSS2:ERG* is also expressed in castration resistant forms of the disease due to reactivation of AR (Cai et al. 2009).

Furthermore*, TMPRSS2:ERG* is more commonly found in poorly differentiated tumours than in well differentiated tumours (Rajput et al. 2007), but evidence also exists to suggest that the *TMPRSS2:ERG* fusion has been associated with disease stage, but does not predict recurrence or mortality amongst men treated with radical prostatectomy (Pettersson et al. 2012). Furthermore, a study in Brazilian patients showed no correlation between ERG expression and clinical and pathological parameters, but suggested that the frequency and specificity of *TMPRSS2:ERG* fusion may aid as a diagnostic tool (Eguchi et al. 2014).

TMPRSS2 is located 3 Mb upstream of *ERG* on human chromosome 21q22.2. The upstream regulatory elements and promoter of the *TMPRSS2* gene drive the over expression of *ERG* upon the formation of the fusion gene (Mani et al. 2011) (Figure 5). The most common fusion is between Exon1 of TMPRSS2 and Exon4 of ERG, occurring in 85% of cases, followed by Exon1-2 of TMPRSS2 fused with Exon4 of ERG (20% of cases). Fusions with Exon2 of TMPRSS2 have been associated with more aggressive forms of PCa (Wang et al. 2006). Evidence exists to suggest that *TMPRSS2:ERG* fusion is an early, or perhaps even initiating event in PCa tumourigenesis, *TMPRSS2:ERG* is detected less frequently in PIN than in PCa, however *TMPRSS2:ERG* fusion events are frequently detected in

PIN lesions that are adjacent to *TMPRSS2:ERG* positive tumours (Perner et al. 2007, Carver et al. 2009).

The mechanism underlying *TMPRSS2:ERG* fusion is not yet fully understood. However, gene fusion can be induced by AR, in both malignant and non-malignant prostate cells. LNCaP cells treated with DHT (the more potent and physiologically relevant testosterone-derivative) for 24 hours induced the *TMPRSS2:ERG* fusion and cells treated for just 3 hours showed co-localization of the *TMPRSS2* and *ERG* genes (Bastus et al. 2010, Mani et al. 2009, Lin et al. 2009). Non-malignant cells (PNT1A and PNT2) were capable of inducing the fusion gene after prolonged treatment with DHT for 5 months, and also showed increased gene proximity after DHT treatment for just 3 hours (Bastus et al. 2010). This is suggestive of an early role for *TMPRSS:ERG* fusion in prostate carcinogenesis.

Population differences in the occurrence of *TMPRSS2:ERG* fusion has also been demonstrated through high-resolution SNP array genomic copy number analysis. Differences in the frequency of the *TMPRSS2:ERG* fusion gene have been described between Chinese and UK-based populations (Mao et al. 2010), as well as western and Asian countries (Lee et al. 2010, Magi-Galluzzi et al. 2011), with significantly higher incidences of *TMPRSS2:ERG* fusion in patients that reside in western countries.

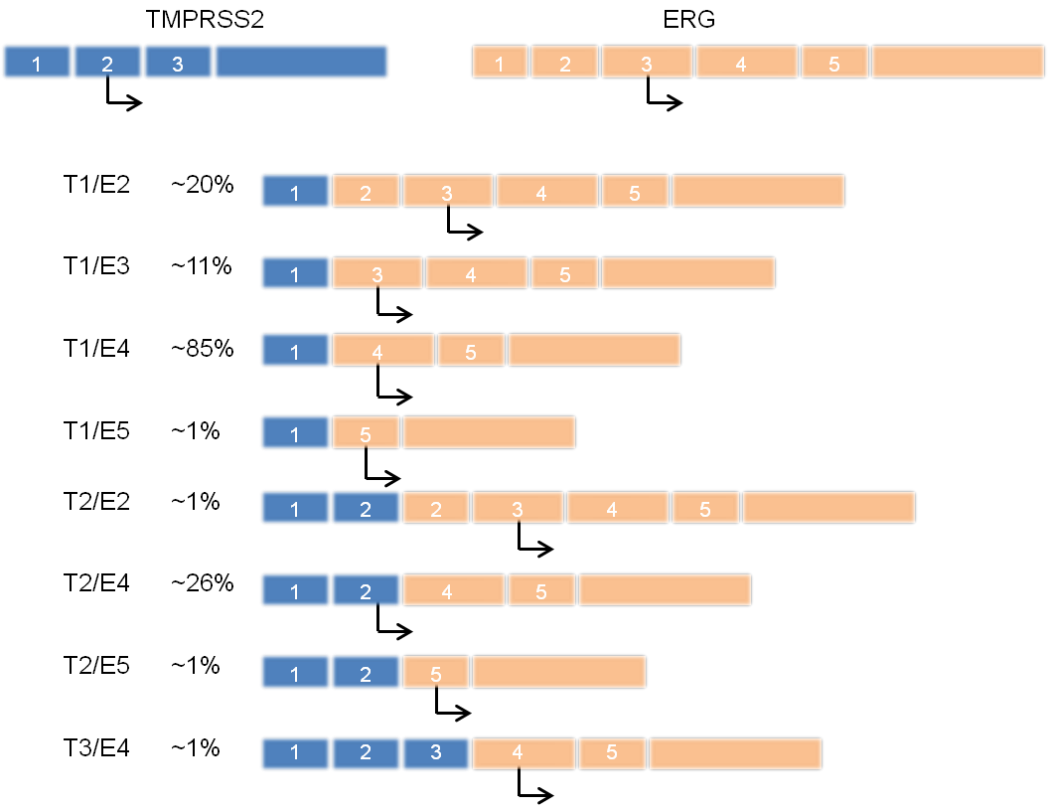


Figure 5 Fusion of the *TMPRSS2* and *ERG* genes found in PCa

Exons (boxes) for TMPRSS2 are shown in blue and ERG in orange. Black arrows indicate the first in-frame translation initiation site in each fusion transcript. Adapted from (Wang et al. 2006).

1.3 Androgen structure, function and role in PCa development and progression

1.3.1 Androgen production and metabolism

AR is a steroid hormone receptor that is required for growth of the normal prostate. Gonadotropin-releasing hormone (GnRH), otherwise known as luteinizing-hormone releasing hormone (LHRH), is synthesised and released by the hypothalamus in a pulsatile fashion (Conn and Crowley Jr 1991). It reaches the anterior pituitary gland, where it stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH then travels to the testicles, where it induces the Leydig cells to produce testosterone. When testosterone arrives at the prostate it is converted by the enzyme 5-α-reductase into 5-α-dihydrotestosterone (DHT) (Figure 6). DHT is an AR agonist that activates an array of AR responsive genes, resulting in normal prostate development (Conn and Crowley Jr 1991).

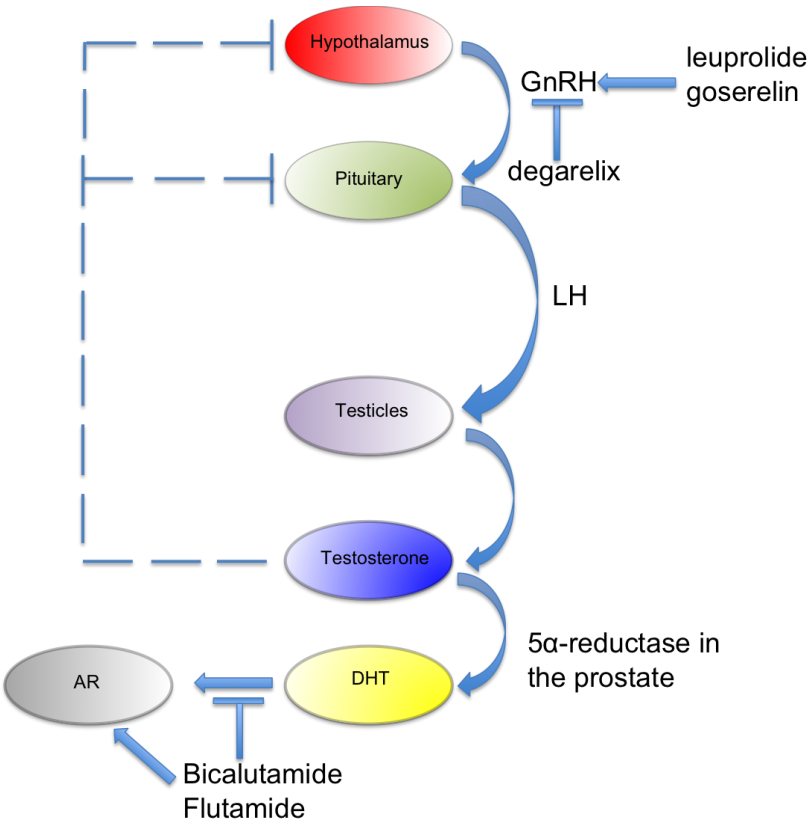


Figure 6 Androgen hormonal feedback

Stimulation is shown by arrows. Negative feedback loop shown by dashed lines. The hypothalamus releases GnRH, which induces the Pituitary gland to release LH. Leuprolide and goserelin act as agonists stimulating the release of more GnRH, degarelix is an antagonist which blocks the production of GnRH. When LH enters the testicles, testosterone is released and feeds back to the hypothalamus and the pituitary gland in a negative feedback loop. Testosterone also travels to the prostate, where it is converted to DHT by 5α-reductase. DHT is then free to bind to AR. Bicalutamide and Flutamide act as AR antagonists and compete with DHT for binding to the AR. GnRH= Gonadotropin releasing hormone; LH= luteinizing hormone; DHT= dihydrotestosterone.

1.3.2 Androgen Receptor biology

Androgen receptor plays a critical role in male sexual differentiation, development and maintenance and is activated by binding of androgenic hormones testosterone or DHT (Roy et al. 1999). AR is a member of the nuclear hormone receptor of transcription factors that responds to androgenic signals from the testes in the form of testosterone/dihydrotestosterone (DHT) (Gaughan et al. 2011). Testosterone is converted to the more active form of androgen, DHT by 5-α-reductase. Following catalysis, DHT binds to AR causing AR homodimerization followed by its translocation into the nucleus. Once homodimerized, AR binds to androgen response elements (AREs) on target genes, contributing to transcriptional activation of prostate specific genes (Culig 2003).

AR is a 110 kDa steroid hormone receptor composed of 919 amino acids, located on the X chromosome at Xq11-12. AR contains four domains; 1) an amino terminal activation domain (NTD), encoded by Exon1. The NTD is the least conserved of the four domains, allowing recruitment of AR co-regulators for activation of prostate specific genes. 2) A DNA-binding domain (DBD), encoded by exons 2 and 3 that is responsible for binding to AREs of prostate specific genes (Shaffer et al. 2004). 3) A hinge region (HiR) encoded by Exon4, containing sites essential for phosphorylation, accetylation and degradation of AR and 4) A ligand-binding domain (LBD) encoded by Exons5-8, central for recognition and binding of androgens (Figure 7). AR contains two activation domains, AF-1, located in the NTD and AF-2 located in the LBD. AF-1 is responsible for the majority of AR transactivation (Bennett et al. 2010), whereas AF-2 mediates NTD/CTD interaction, which has been shown to interact with a number of co-regulators and is important in stabilising bound ligand (Saporita et al. 2003).

The AR consists of 12 alpha helices that undergo conformational changes in order to provide an interface for the recruitment of nuclear co-regulator proteins essential for controlling the activation of androgen-dependent genes via binding to AREs (Gaughan et al. 2011). These genes are involved in important processes, for example angiogenesis and apoptosis, and the interface is created when the 12 alpha helices line up creating a closed conformation.

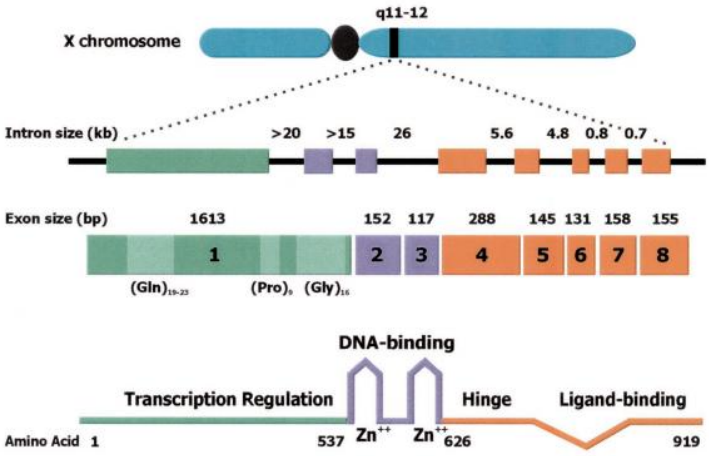


Figure 7 Genomic position of the AR gene

The AR gene spans 80 kb with intron and exon regions shown in the second panel. The three codon repeat regions of the first exon are shown, these constitute the N-terminal domain and this region is responsible for transcriptional activation. The DNA binding domain is located adjacent to the N-terminal domain. The ligand binding domain is

adjacent to the hinge region. This is the region that many AR agonists bind to in competition with DHT. From (Gelmann 2002)

1.3.3 Role of AR in metastatic castration resistant PCa

Androgen signalling plays a large role in PCa development and is therefore the initial target for therapeutic intervention (Culig et al. 2000). Decreasing the levels of circulating androgens by chemical castration frequently results in regression of tumours (see section 1.4.4.1). However, recurrence of castration resistant PCa eventually occurs (Shen and Abate-Shen 2010). Castration resistant tumours express AR as well as AR target genes, for example PSA, suggesting that pathway activity is intact (Gao, Arnold, and Isaacs 2001). Mechanisms described for this signalling have included amplification of AR gene copy number (Visakorpi et al. 1995), gain of function mutations of AR that may confer increased protein stability and sensitivity to androgens (Taplin et al. 1995), and expression of alternative splice isoforms that encode a constitutively active AR (Figure 8) (Dehm et al. 2008). Androgen synthesis and conversion of weaker androgens to testosterone and DHT, by the endogenous expression of androgen synthetic enzymes from the tumour tissue have also been reported to cause increased AR signalling (Stanbrough et al. 2006). Genomic alteration studies have identified a number of somatic mutations that can influence the outcome of cancer. The AR gene is the most commonly mutated steroid hormone receptor gene, with more than 660 mutations identified so far (Koochekpour 2010). Mutations in AR are found in 25-50% of androgen-independent (AI) and metastatic PCas in comparison to around 2% of untreated localised PCas (Gottlieb et al. 2004, Linja and Visakorpi 2004, Koochekpour 2010). Over 70 different somatic missense mutations have been described in PCa patients, yet only a few have been studied. The first AR mutation was described in the LNCaP PCa cell line (Veldscholte et al. 1990). The T877A mutation occurs in the ligand binding domain of AR, resulting in decreased ligand specificity and promiscuous binding and activation of AR by non-androgenic steroids.

It is currently difficult to pinpoint the exact time at which PCa progresses to castration resistance. It is thought that castration resistant cells arise through genetic/epigenetic conversion of androgen-dependent cells during androgen deprivation (Shen and Abate-Shen 2010). Another model proposes that the cells arise from a population of rare, castration resistant cells within an androgen-dependent tumour. It is suggested that androgen deprivation allows for the

selection of these cells due to elimination of sensitive cells (Isaacs and Coffey 1981, Tso et al. 2000, Feldman and Feldman 2001).

It is this castration resistant form of the disease that is increasingly difficult to treat, 5 year survival rates for men at this advanced stage are 33% compared to 100% survival rates for localized disease (eMedTV July 2013).

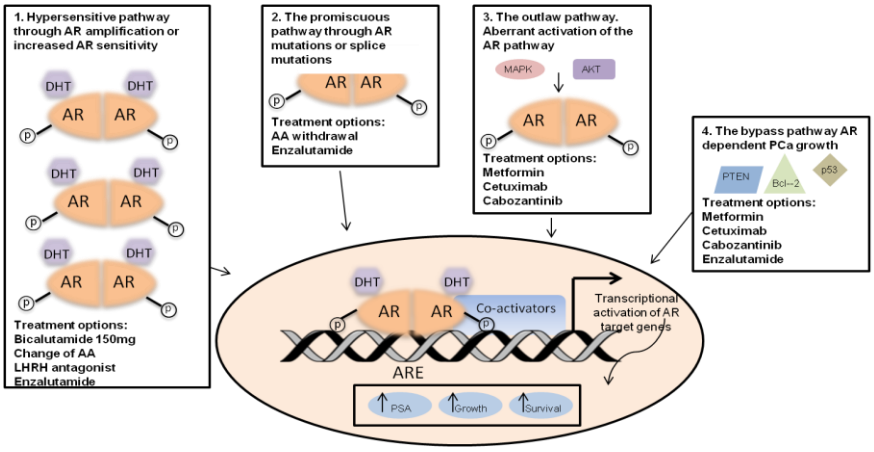


Figure 8 Mechanisms of androgen receptor gain of function in castration resistant PCa

1. Upregulation of AR through gene amplification or enhanced sensitivity to AR. In addition more testosterone is converted to DHT. **2**. The specificity of AR is altered, allowing activation by non-androgenic molecules that are already present in the circulation. **3**. AR is phosphorylated through an alternative pathway either by AKT (protein kinase B) or the mitogen-activated protein kinase pathway (MAPK) resulting in ligand independent activation of AR. **4**. Parallel survival pathways negate the need for AR or its ligand e.g. the anti-apoptotic protein BCL2 (B-Cell lymphoma 2). Adapted from (Feldman and Feldman 2001).

1.3.4 AR Promoter and enhancer- transcriptional control, coactivators and corepressors of AR

Following its activation, AR binds to AREs on gene promoters and enhancers, where it recruits co-regulators including coactivators and corepressors that orchestrate chromatin remodelling and transcriptional regulation. The number of coactivators and corepressors now exceeds 200. AR dependent gene expression is therefore regulated by relative levels of AR coactivators and corepressors that modulate AR activity in response to changing hormone levels. These transcriptional regulators do not typically interact with the DNA, but co-operate with AR at target gene promoter and/or enhancer regions for four purposes: 1) to assist DNA binding, 2) to induce chromatin remodelling and accetylation/deacetylation of chromatin through recruitment of histone acetyltransferases (HAT) and histone deacetyltransferases (HDACs) respectively,

3) to control cofactor binding to the AF-2 coactivators binding groove through regulation of AR N/X interaction and finally 4) to recruit transcription factors that form the pre-initiation complex (PIC).

1.3.4.1 Promoter and enhancer transcriptional control

Maintaining control of gene function is essential for the development of all organisms, little is known about how the correct patterns of gene activation are maintained genome-wide, due to the complexity of this process. Genes lie adjacent to each other and many have multiple differentially regulated transcripts. Additionally, chromatin is arranged in a three-dimensional fashion, bringing genes that are located on different chromosomes into close proximity in order to interact with one another (Atkinson and Halfon 2014).

The core promoter is typically located 35-40 bp upstream or downstream of the transcription start site (TSS). This sequence is usually sufficient to activate gene expression in a reporter assay and is required for transcription by eukaryotic RNA polymerase II. Basal transcription machinery, essential for promoter activation, interacts with core motifs found within the core promoter (Zhu and Halfon 2009). Identification of these core motifs is hindered by the fact that there are no universal motifs that are common to all promoters and no universally required element within promoters necessary for promoter activity. Despite this, the most common promoter motif is the TATA box, which binds the TATA-box binding protein (TBP) and is present in 5-20% of mammalian promoters (Cooper et al. 2006, Gershenzon and Ioshikhes 2005). The TATA box consists of a canonical sequence, typically TATAAAAA, but many variations of this sequence have been found and this sequence has been conserved throughout evolution, further demonstrating its importance in promoter activation (Patikoglou et al. 1999).

In order to fully understand promoter activation many attempts have been made to use large-scale mapping of TSSs, that have revealed different classes of promoters based on TSS distribution (Carninci et al. 2006, Hoskins et al. 2011). These can be grouped into single or broad. Single peak promoters span only one or several bp, whereas broad promoters can span up to 100 bp, further complicating the identification of gene specific promoters. Additionally a role in gene regulation has been suggested for the extended promoter region, up to 350 bp upstream of the TSS (Cooper et al. 2006).

In addition to the complex nature of promoter regions, enhancers also play a vital role in the activation of specific genes. Transcriptional enhancers are distal non-coding sequences that positively regulate transcription, regardless of orientation or distance in relation the gene being transcribed (Banerji, Rusconi, and Schaffner 1981) and have been known to be able to activate transcription of genes located on a different chromosome (Geyer, Green, and Corces 1990, Lomvardas et al. 2006). The complex regulation of a gene can be controlled by multiple enhancers, up to a few hundred base pairs in length. The main function of an enhancer is to act as a platform on which activators, repressors and chromatin modifying enzymes can bind to modulate gene expression (Atkinson and Halfon 2014). Upstream/downstream enhancers are thought to interact with DNA through chromatin looping, resulting in the stabilization of RNA polymerase binding or release of stalled polymerase. Evidence that enhancers are marked by specific sets of histone modifications is mounting, monomethylation of histone 3 lysine 4 (H3K4me1) and accetylation of histone 3 lysine 27 (H3K27ac) are hallmarks of enhancers and have been used to predict the position of enhancers in a number of human cell lines, identifying over 400,000 enhancers. However, the majority of these have not been confirmed *in vivo* (Dunham et al. 2012).

Some enhancers are known to solely interact with specific promoters (Butler and Kadonaga 2001). Whereas others have been shown to interact with similar promoters but not dissimilar promoters in zebrafish (Gehrig et al. 2009). Evidence also exists to suggest that enhancers are not gene specific and can interact with a number of different promoters (Kermekchiev et al. 1991). This suggests a more complex role for enhancers in gene specific activation. Additionally, in order to facilitate/prevent promoter/enhancer interaction coactivators and corepressors must bind to specific regions on promoters and enhancers to support/prevent interaction. A number of gene specific coactivators and corepressors have also been identified, which support gene specific activation.

1.3.4.2 AR coactivators

When a cell is not undergoing DNA replication the genomic DNA is tightly packed into chromatin, generating a barrier for transcriptional activities. In order for AR to stimulate transcription, it must open the chromatin structure to facilitate assembly of the PIC. AR fulfils this task by binding coactivators. These are specific proteins that are generally part of larger complexes that implement specific functions

essential for transcription. Two mechanisms exist to open up the chromatin structure. This first relies on covalent modification of histones thorough (de)acetylation, (de)methylation, (de)phosphorylation, ubiquitnation and sumoylation (Rosenfeld, Lunyak, and Glass 2006). Consequently, the modifications loosen or tighten the DNA-histone interactions by changing the net charge of the nucleosome (Heemers and Tindall 2007) (Figure 9).

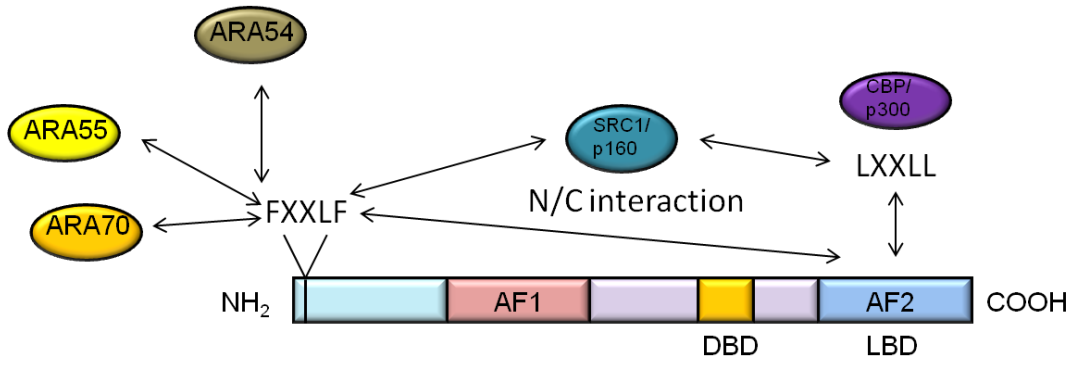


Figure 9 Schematic demonstrating AR coactivator interaction with AR

AR contains a DNA-binding domain (DBD) and activation function 1 (AF1) region in the NH2-terminal region. The AR FxxLF motif interacts with ARA54, ARA55 and ARA70 as well as SRC1/p160. SRC1/p160 also interacts with the AF2 domain of AR through its LxxLL domain. The AF2 domain additionally interacts with the FxxLF motif in the NTD, generating the androgen-dependent N/C interaction.

The most well understood of the histone modifiers are the histone acetyltransferases (HATs), which catalyze the addition of an acetyl group to specific lysine residues, opening up the DNA. The best characterised AR coactivators are the p160/SRC-family of proteins, together with p300 and CBP (Table 4).

In fact, immunohistochemical studies have shown that SRC1 expression is increased by 50% in androgen-dependent PCa samples compared to benign or normal prostate tissues. This figure increased to 63% in CRPC samples (Gregory, He, et al. 2001). The p160 coactivators interact with the AR NTD and LBD and directly influence AR transactivation via their histone acetyltransferase activity. These coactivators also act indirectly by acting as a platform for the binding of secondary coactivators such as p300. Interestingly, p300 levels were also found to correlate with proliferation *in vivo* and were associated with larger tumour volumes. *In vitro,* levels of p300 have increased under conditions of androgen deprivation, offering a growth advantage in androgen insensitive cells (Heemers et al. 2007).

P300/CBP functions as a coactivator through bridging of DNA transcription factors to the basal transcriptional machinery whilst simultaneously acting as a scaffold for the nucleation of various transcription factors. It acetylates all four histones at a specific lysine residue on the histone tails, this process loosens histone-DNA interaction through neutralization of the negative charge (Iyer, Ozdag, and Caldas 2004).

Table 4 Summary of the major AR coactivators involved in transcriptional activation of AR dependent genes.

Coactivator	Function	AR binding site	Effect on AR signalling
CBP/p300	• Bridges DNA transcription factors to the basal transcription machinery • Posses HAT activity	AF2 via the LxxLL motif.	Renders chromatin more easily accessible to AR and transcription factors, increasing AR signalling
p160	• Acetylates histones to unfold chromatin for access • Acts as platform for binding of secondary coactivators	AF2 via the LxxLL motif. AF1 via FxxLF motif	Renders chromatin more easily accessible to AR and transcription factors, increasing AR signalling
SRC1	• Nuclear receptor coactivator	AF1 or AF2 via LxxLL motif	
ARA54	• AR coactivator	FxxLF interaction with LBD not through AF2	Enhances AR-dependent transcriptional activation
ARA55	• Role in stromal-epithelial interaction in development • AR coactivator	FxxLF interaction with LBD not through AF2	Modulates AR specificity in response to agonists/antagonists
ARA70	• Potentiates AR transactivation in the presence of androgen • AR coactivator	FxxLF interaction with LBD not through AF2	Enhances AR-dependent transcriptional activation
SWI/SNF	• Alters the chromatin structure through remodelling	N/A	Allows AR access to AR binding sites

Binding to coactivators takes place through a short helical motif, LxxLL, which binds to a groove on the LBD. This groove is formed by charged and hydrophobic residues in helices 3, 4, 5 and 12. The three leucine residues of the structure are orientated towards the LBD surface and line one face of the helix, with the first and last leucine residues embedded within the hydrophobic groove. This binding is further stabilized by hydrogen bonds between the peptide backbone and charged

residues at the opposing end of the groove. Several coactivators contain multiple LxxLL motifs for binding, however, AR interacts relatively weakly with LxxLL motifs in many AR coactivators. AR preferentially binds to related FxxLF sequences, these were initially identified in the AR NTD (He, Kemppainen, and Wilson 2000), and were later shown to play a crucial role in the recruitment of AR cofactors ARA54, ARA55, ARA70 and hRAD9 (He et al. 2002, Wang et al. 2004). Binding through FxxLF motifs modulates the association of the AR NTD with the carboxy terminal domain (N/C) and interacts with the hydrophobic groove in the C-terminal AF-2 domain. Moreover, FxxLF motifs are unable to bind to other nuclear receptors, suggesting that cofactors with FxxLF sequences will preferentially bind to AR (Dubbink et al. 2006).

The second method of regulating chromatin structure is through chromatin remodelling, utilising large coactivator complexes such as SWI/SNF, WINAC and NUMAC. These complexes can utilise the energy derived from ATP hydrolysis to alter the stability of the nucleosomes (van de Wijngaart et al. 2012). These chromatin remodelling complexes always contain a catalytic subunit belonging to the SNF2 family of ATPases, usually BRG1 or BRM along with 10-12 BRG1-associated factors (Figure 10).

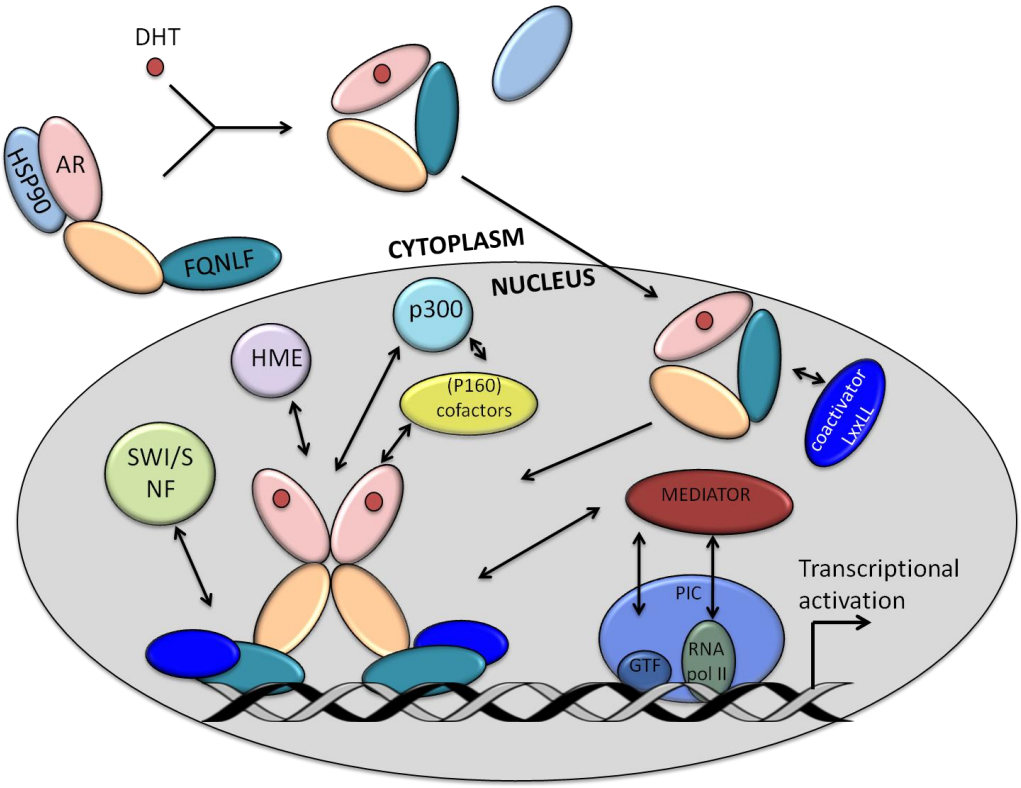


Figure 10 Mechanisms of AR coactivator action

Upon ligand binding to AR, interaction takes place between the N/C terminals. This interaction is disrupted upon binding to AREs on the DNA, allowing recruitment of cofactors. This process results in accumulation of various complexes that modulate chromatin and initiate transcription regulation. AR= androgen receptor, HSP= heat shock protein, AREs= androgen response elements, RNA PolII= RNA polymerase II, DHT= dihydrotestosterone, GTF= general transcription factor, PIC= preinitiation complex. Adapted from (van de Wijngaart et al. 2012).

1.3.4.3 AR corepressors

Whilst a number of genes are upregulated by AR expression, some genes are downregulated, suggesting that AR can also function as a transcriptional repressor (see section 1.3.5). More recent evidence has suggested that AR does not directly repress transcription, but that downregulation of target genes is due to the binding of corepressors. These corepressors most commonly function through: 1) inhibiting DNA binding and coactivator binding, 2) regulation of AR N/C interaction, 3) abrogation of AR-chromatin association or nuclear translocation and 4) playing an active role in condensing the chromatin to its inactive state (Figure 11).

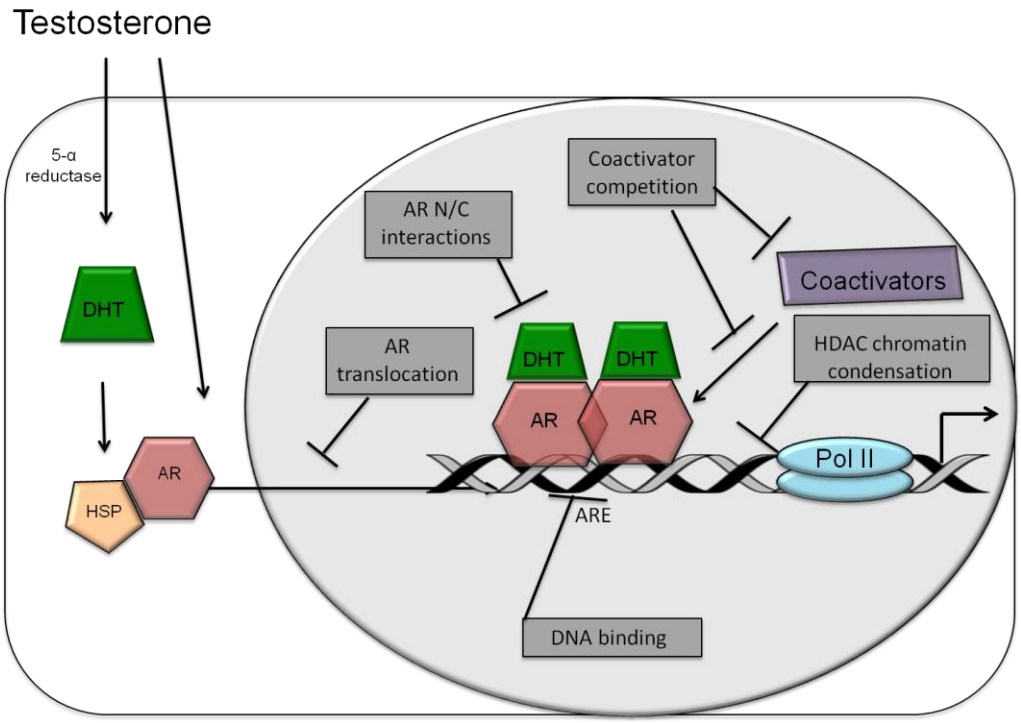


Figure 11 Mechanisms of AR corepressor action

Binding of AR to AREs on the DNA, and subsequent coactivator recruitment is essential for transcription of AR dependent genes. AR corepressors can interfere with AR activity through at least four differing mechanisms; 1) inhibition of AR nuclear translocation, 2) Inhibition of N/C interaction, 3) inhibition of DNA biding and finally 4) inhibition of coactivator recruitment. AR= androgen receptor, HSP= heat shock protein, AREs= androgen response element, HDAC= histone deacetylase, PolII= RNA polymerase II. Adapted from (Burd, Morey, and Knudsen 2006).

The most well understood AR corepressors are nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid (SMRT), that repress transcription through Sin3 and recruitment of HDACs. Additionally, these corepressors disrupt AR N/C interaction and compete with SRC/p160 AR coactivators. Upon ligand binding, NCoR and SMRT function as huge scaffold proteins that are recruited by AR, requiring both the LBD and NTD and an extended LxxLL like motif, termed corepressor nuclear receptor (CoRNR) (Hu and Lazar 1999). Additional AR corepressors such as ErbB3-binding protein (EBP1), DJ1-binding protein (DJBP) and TG-interacting factor (TGIF) utilise Sin3 complexes, consisting of Sin3a and Sin3b together with either HDAC1 or HDAC2 to modulate transcriptional repression. Most importantly, EBP1 is recruited to AR targets in the presence of therapeutic antagonists together with HDAC2.

As mentioned previously, ligand binding induces the binding of the FxxLF motif to the hydrophobic surface of the AR LBD. Corepressors have been shown to

interfere with this N/C interaction through multiple mechanisms. Firstly they can compete for binding to prevent the formation of N/C interaction, the FxxLF domain within hRad9 has been shown to compete for binding to the AR LBD, blocking AR activity as a result (Wang et al. 2004). Secondly, corepressors can bind directly to regions of AR that mediate N/C interactions, this is the case for large tumour suppressor 2 (lats2), which binds directly to a residue on the LBD of AR that is essential for N/C interaction (Powzaniuk et al. 2004). Finally, a number of corepressors, including SMRT, block N/C interaction through an unknown mechanism. Thus, these examples of targeting N/C interaction highlight a major pathway in which corepressors function to inhibit AR transcription.

A small group of corepressors can prevent AR translocation to the nucleus and subsequent binding to the DNA. One example of which is the phosphatase and tensin homolog (PTEN) deleted on chromosome 10, which sequesters AR in the cytoplasm in LNCaP cells, although the mechanism by which this occurs is currently unknown (Lin et al. 2004). p21-activated kinase (PAK6) has also been shown to inhibit nuclear translocation through interaction with the DBD (Schrantz et al. 2004) and Rack1 has been associated with a decline in AR promoter occupancy due to its activation of the protein kinase C pathway (Rigas et al. 2003).

A small number of corepressors can also compete with coactivators for recruitment to the AR complex, through interaction with the coactivator, or AR. NCoR and SMRT have been shown to function by binding to AR. In fact, Yoon et al showed that ablation of either of these corepressors leads to enhanced recruitment of steroid receptor coactivator 1 and p300 (Yoon and Wong 2006). Conversely, corepressors can modulate coactivators to prevent binding to AR in a number of ways, for example: GSK-3β targets the ß-catenin AR coactivator specifically for degradation (Masiello et al. 2004) and proline-rich tyrosine kinase 2 (PYK2) can phosphorylate the ARA55 coactivator, preventing AR association (Wang et al. 2002).

Finally, there are a group of corepressors that function to inhibit AR transactivation through unknown mechanisms. An example of such a corepressor is amino-terminus enhancer of split (AES), which is known to interact with TFIIE. However, the relevance of this is not yet understood. A second family of corepressors are the protein inhibitor of activated STAT (PIAS). A number of members of this family

act as coactivators, but have also been found to function as corepressors. They are thought to modulate AR function by post-translational modification through sumoylation of AR (Schmidt and Müller 2003).

In summary, AR corepressors can function to impede AR transactivation through a number of mechanisms, the deregulation of these corepressors in PCa could potentially offer new therapeutic avenues for the treatment of cancer, due to the importance of AR regulation. The major AR corepressors and their function are listed in Table 5.

Table 5 Summary of the major AR corepressors involved in restricting expression of AR dependent genes.

Corepressor	Function	AR binding site
Iats2	• Disrupts AR N/C interaction	LBD of AR
PAK6	• Inhibits nuclear translocation through interaction with the DBD	N/A
PTEN	• Sequesters AR in the cytoplasm in LNCaP	N/A
GSK-3β	• Targets β-catenin AR coactivator for degradation	N/A
PYK2	• Phosphorylates ARA55 preventing AR association	N/A
SMRT/ NCoR	• Binds to and sequesters AR	Binds to LBD of AR through CoRNR boxes

CoRNR= corepressor nuclear receptor boxes

1.3.5 Androgen regulated genes and prostate specificity.

Androgen responsive/regulated genes are characterised by significantly altered expression levels upon androgen treatment and are essential for prostatic development through proliferation, differentiation, cell cycle and apoptosis (Vaarala et al. 2012)**.** Consequently, the identification of androgen responsive genes can lead to novel targets for PCa treatment and biomarkers to detect and monitor disease progression.

Over the last decade, researchers have undertaken genome-wide analysis to identify androgen-responsive genes, resulting in the identification of an unparalleled number of genes differentially regulated by AR, that reaches well into the thousands with the introduction of microarray analysis (Jin, Kim, and Yu 2013). Androgen responsive LNCaP cells have been critical to these studies in order to identify differentially regulated genes before and after androgen stimulation. Whilst the majority of research has focussed on those genes that are upregulated by

androgen receptor stimulation, a more complex global expression profile is emerging, whereby androgen receptor stimulation can both up-regulate (DePrimo et al. 2002, Velasco et al. 2004) or down-regulate genes (Ngan et al. 2009), suggesting a more composite role for AR in prostate carcinogenesis.

AR is a hormonal transcription factor, activated by binding of androgen, resulting in translation of AR protein, a DNA binding protein that binds to cis-regulatory elements in androgen responsive genes. Upon ligand binding, AR undergoes a conformational change, where heat shock proteins dissociate and AR becomes phosphorylated, leaving AR free to translocate to the nucleus and homodimerise (van Royen et al. 2012). In the nucleus AR binds to AREs located along the DNA of specific genes (Figure 12). AREs consist of a DNA binding motif comprising of 15 nucleotides, this motif is an imperfect palindrome separated by 3 nucleotides (AGAACAnnnTGTTCT). Despite the general palindromic sequence, functional AREs can differ from the consensus sequence by several nucleotides (Khorasanizadeh and Rastinejad 2001). Binding of AR is also tightly regulated by a number of transcriptional coactivators including GATA2, OCT (Wang et al. 2007) and FOXA1 (Gao et al. 2003) and growing evidence suggests that AR primarily binds to distal enhancers, interacting with promoters through chromatin looping (Wang, Carroll, and Brown 2005, Wu et al. 2014). These enhancers can range from several, to hundreds of kb away from the gene specific promoters with which they interact.

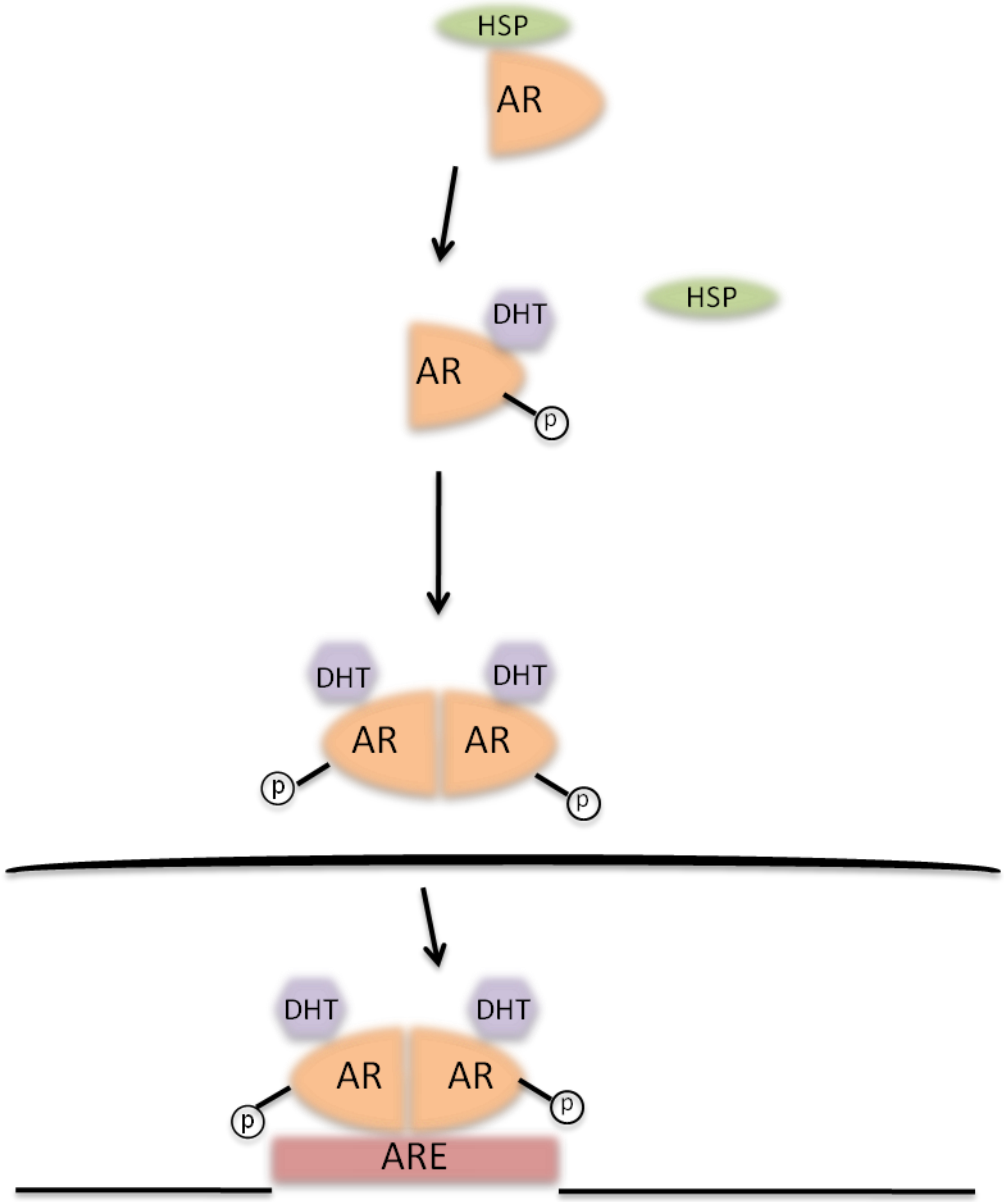


Figure 12 Activation of AR target genes

Upon binding of DHT, HSP dissociates from AR, leaving AR free to homodimerise and enter the nucleus. Within the nucleus AR homodimers bind to AREs on the DNA to activate transcription of AR dependent genes. AREs = Androgen Response Elements, HSP= Heat Shock Protein.

1.4 PCa treatment strategies

1.4.1 Overview of PCa treatment

Due to the heterogeneity and indolent nature of PCa, selection of the most suitable treatment to limit patient mortality and ensure quality of life remains a fundamental challenge in tackling the disease. Treatment strategies are dependent on age of the patient, cancer stage and grade and the overall health of the patient. An overview of PCa treatment strategies can be seen below (Figure 13).

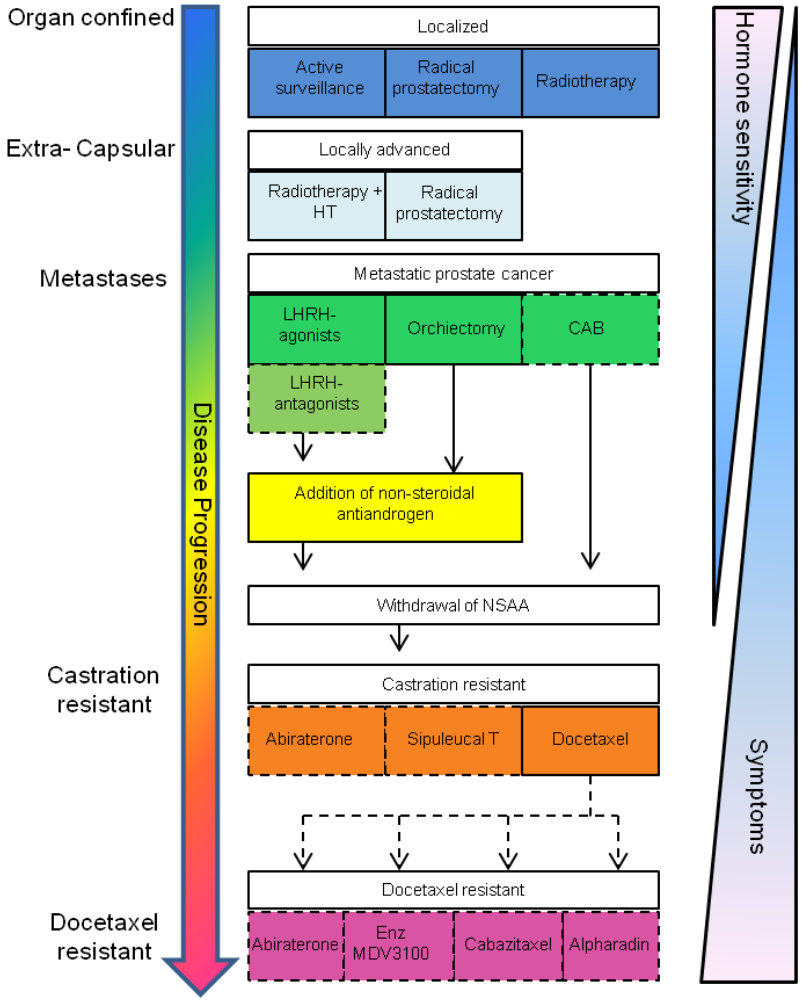


Figure 13 Overview of PCa therapy

This diagram shows the standard therapy for each stage of PCa. Therapeutic options are dependent on PCa grade and stage, patient age, general health and personal preference. Based on EAU guidelines the treatments in the dotted frame are not considered standard therapy. HT= hormone therapy, ADT= androgen deprivation therapy, CAB= combined

androgen blockade, NSAA= non-steroidal antiandrogen. Adapted from (Helsen et al. 2014).

1.4.2 Localised PCa treatment.

Localised PCa is often left untreated in the UK, in order to avoid 'over treatment'. Depending on the age and general health of the patient, doctors may choose to monitor the patient by 'watchful waiting'. This is most often the case in men who are diagnosed with PCa, but are presenting with no symptoms, or who have other medical problems that render treatment unsuitable. Patients with localised cancer can also be monitored by 'active surveillance' (AS). This is undertaken by regular PSA testing, monitoring the size of the prostate and evaluation of Gleason score by biopsies. Disease progression is monitored to allow for radical treatment at a later stage (Parker 2004). A study monitored a cohort of 450 patients with clinical stage T1c or T2a, PSA <10 ng/ml, Gleason score ≤6 and patients over the age of 70 with a Gleason score <7 and PSA up to 15 ng/ml, not all patients had been diagnosed with the disease (Klotz et al. 2010). The overall survival rate was 78.6% at 6.8 years witht the prostate cancer specific survival rate at 97%. Additional studies have demonstrated a low rate of progression and disease specific death in patients that have been carefully selected to undergo AS due to low risk (Klotz 2010). Overall AS is a well accepted strategy for the treatment of low-risk localised PCa. However, the many benefits of this strategy, including lower costs to the healthcare system, reduction of the unwanted side effects of surgery and better quality of life must be carefully weighed against the risks involved in delaying therapy. This calls for more stringent measures to assess the risk of localised PCa to the patient including more reliable biomarkers to distinguish aggressive from indolent forms of the disease.

Methods to treat localised PCa include radical prostatectomy, brachytherapy, and radiotherapy as well as hormonal therapies, including androgen ablation, all of which produce adverse side effects including incontinence, bleeding and erectile dysfunction (Gomella, Johannes, and Trabulsi 2009). Men who do undergo prostatectomy in comparison to no treatment are more likely to suffer from erectile dysfunction (80% vs 45%) and urinary leakage (49% vs 21%) over a 5-year period (Bergman and Litwin 2012). Approximately 25% of patients present with localised PCa at diagnosis. If receiving treatment most are given single-modality treatments including radical prostatectomy or radiotherapy. There are a number of types of radical prostatectomy, including: radical retropubic prostatectomy, laparoscopic

radical prostatectomy and robotic-assisted laparoscopic prostatectomy (Ficarra et al. 2009). Localised and locally advanced cancer can also be treated by androgen deprivation therapy, which aims to reduce the levels of circulating testosterone that are responsible for growth of PCa. This treatment can be utilised at all stages of the disease, however this often leads to more aggressive, incurable forms of PCa known as 'castration resistant', a term used to describe PCa that is unresponsive to hormonal treatment, due to the sustained activation of the AR signalling pathways (Gao, Arnold, and Isaacs 2001). Patients that present with higher Gleason scores and rising PSA levels often benefit from a combinational therapy including ADT in conjunction with radiotherapy (Heidenreich 2012).

1.4.3 Metastatic castration resistant PCa

The major cause of patient mortality is due to the propensity for PCa to metastasize to the lymphatic tissue and the bone, conveying osteoblastic rather than osteolytic lesions (Bubendorf et al. 2000). Metastatic PCa is a debilitating disease that includes symptoms such as bone pain, spinal cord compression, renal failure and anaemia. Little is known about this mechanism of metastasis, partly as there have been inadequate mouse models for metastatic PCa and also due to unavailability of human metastatic samples (Shen and Abate-Shen 2010). The presence of circulating tumour cells in the blood stream and the bone marrow have been shown, yet these have not obtained full metastatic capabilities. These cells do, however, show multiple chromosomal rearrangements typical of those found in advanced PCa (Holcomb et al. 2008). Treatment for metastatic PCa usually involves the combination of androgen deprivation therapy with cytotoxic drugs. Chemotherapy is mainly palliative and reduces the symptoms, but does not cure the disease. Chemotherapy drugs that are most commonly used to treat PCa include docetaxel (Taxotere) and mitoxantrone (Novantrone).

1.4.4 Targeting AR for PCa therapeutics

1.4.4.1 Medical and chemical castration

As PCa begins to advance, treatments include hormone therapy, radiotherapy alone, or radiotherapy in combination with hormone therapy and radical prostatectomy. Whilst radical prostatectomy and radiotherapy are still the most effective treatments for locally advanced PCa, adverse side effects including

erectile dysfunction, infertility and incontinence make less invasive procedures more attractive.

By inhibiting the release of androgens, prostate cells are deprived of proliferative stimulation, and as a result undergo apoptosis. There are several methods of inducing androgen deprivation that are used for the treatment of locally advanced androgen-dependent PCa that include: castration (surgical or chemical), antiandrogens and androgen blockade (Chen, Clegg, and Scher 2009). Surgical castration involves the removal of the stimulatory input of testosterone in the body by orchiectomy; complete removal of both testicles. However, the development of GnRH agonists and antagonists resulted in a non invasive method of inducing castrate levels of testosterone in the body, known as chemical castration. Chemical castration involves the use of GnRH agonists or antagonists that block the messenger processes to the brain, and prevent the release of testosterone.

GnRH agonists work by continuous stimulation of the pituitary gland, overcoming the pulsatile release of GnRH from the hypothalamus in order to shut down the production of LH. Typically used GnRH agonists include leuprolide, usually administered as an intramuscular injection and goserelin, usually administered subcutaneously into the abdomen. After the initial administration of GnRH agonists, testosterone levels can temporarily rise 'flare', resulting in increased prostate cell growth. This growth may cause an increase in patient symptoms; therefore the rise in testosterone is often blocked by co-administration of a nonsteroidal androgen receptor antagonist. In order to avoid the initial flare in testosterone levels, GnRH antagonists can be used to directly block GnRH and instigate a fast reduction in testosterone (Crawford and Hou 2009). The most commonly used GnRH antagonist is degarelix, which is administered subcutaneously as 2 injections (Xu, Jiang, and Wu 2012). Men who have undergone castration either by surgical or chemical means have 90-95% less testosterone in their bodies than normal healthy males.

1.4.4.2 Antiandrogens.

Current hormonal therapies for PCa treatment are not capable of completely inhibiting AR. Changes that occur in AR during cancer progression, for example mutation and over-expression, result in resistance to castration. Therefore, methods of inhibiting AR transcriptional activation by using antagonists that compete with endogenous androgens for binding to the ligand binding domain

(LBD) of AR have been developed (Chen, Clegg, and Scher 2009) (Figure 14). Antiandrogens can be either steroidal or non-steroidal. Steroidal antiandrogens include progesterone analogues and cyproterone acetate (CPA). CPA is non-specific and can activate the glucocorticoid, mineralcorticoid and progesterone receptor, thus it is not currently used as a first line treatment. Non-steroidal antiandrogens were developed in the 70's as a means of avoiding the off-target side effects of steroidal antiandrogens. The most commonly used antiandrogens are bicalutamide, nilutamide and flutamide (Chen, Clegg, and Scher 2009). The exact mechanism by which antiandrogens bind to and inhibit AR has not yet been fully elucidated and appears to be more complex than just competitive binding for AR with DHT. Bicalutamide was approved for use by the Federal Drug Administration in 1995. It is given as an oral dose of 150 mg/day for monotherapy, although when given as a monotherapy patients median survival was 6 weeks shorter in comparison to castration (Tyrrell et al. 1998). Despite this, the American Society of Clinical Oncology recommend treatment with Bicalutamide monotherapy as an alternative for the gold standard ADT and it is the most widely used antiandrogen for treatment of androgen responsive PCa. Bicalutamide has a relatively low affinity for AR, at least 30-fold less than DHT, suggesting that modifications to improve ligand binding could improve AR antagonism (Kolvenbag, Furr, and Blackledge 1998). Nilutamide is only used for concomitant therapies and as there are currently no trials published that use nilutamide as a monotherapy it is not currently registered for use as one (Helsen et al. 2014).

1.4.4.3 Novel antiandrogens

Due to the eventual clinical failure of current antiandrogens, much emphasis has been placed on the development of novel AR antagonists. Enzalutamide (Enz), formally known as MDV3100, is a thiohydantoin derivative with a higher binding affinity for AR than Bicalutamide. As well as binding directly to AR, Enz also inhibits AR function by blocking nuclear translocation and DNA binding (Tran et al. 2009). In 2012 Enz was approved by the FDA after the AFFIRM trial demonstrated that Enz treatment increased overall survival in patients who progressed after docetaxel treatment in comparison to the placebo treated group (Ning et al. 2013, Scher et al. 2012). Patients also benefited from significant improvements in quality of life, including pain palliation and the drug was well tolerated, with limited side effects. A larger Phase III PREVAIL trial was conducted between September 2010 and September 2012 in 1717 men with metastatic castration resistant PCa, who

had not received chemotherapy previously. Interim results showed that Enz treatment improved overall survival by 29% and reduced the risk of radiographic progression of disease by 81%. Due to this promising outcome, the trial was stopped prematurely and all patients on the placebo were offered Enz (Beer et al. 2014). This novel antiandrogen offers an exciting new treatment for men with metastatic castration resistant PCa that is effective in chemo treated patients who have progressed.

1.4.4.4 Steroid biosynthesis inhibitors

Traditional therapies for PCa treatment have focused on the development of drugs that target the AR pathway by direct inhibition or decreasing levels of circulating testosterone. However, these are not effective at completely eradicating serum testosterone levels, due to androgens produced in the adrenal glands. Therefore, newer therapies have focused on targeting the androgen biosynthesis pathway. Abiraterone specifically inhibits CYP17A1, the enzyme responsible for catalysing the conversion of the adrenal androgen precursors pregnenolone and progesterone into DHEA-S and androstenedione (Figure 14). These weak androgens are later synthesised into testosterone and DHT in peripheral tissues and PCa tumours (Cai and Balk 2011).

More recently, abiraterone has also been shown to bind and inhibit AR (Richards et al. 2012). Abiraterone is a derivative of progesterone; its main function is inhibition of CYP17A1, therefore inhibiting the conversion of pregnenolone and progesterone and the subsequent formation of dehydroepiandrosterone (DHEA) and androstenedione, precursors of testosterone. Abiraterone has demonstrated clinical efficacy in both chemo treated and chemo-naive patients, resulting in prolonged progression free survival, prolonged overall survival and delayed clinical decline of patients (de Bono et al. 2011, Ryan et al. 2013). Despite promising results in abiraterone treated patients, PCa continues to progress due to continued AR pathway activation, possibly due to increased AR mutation.

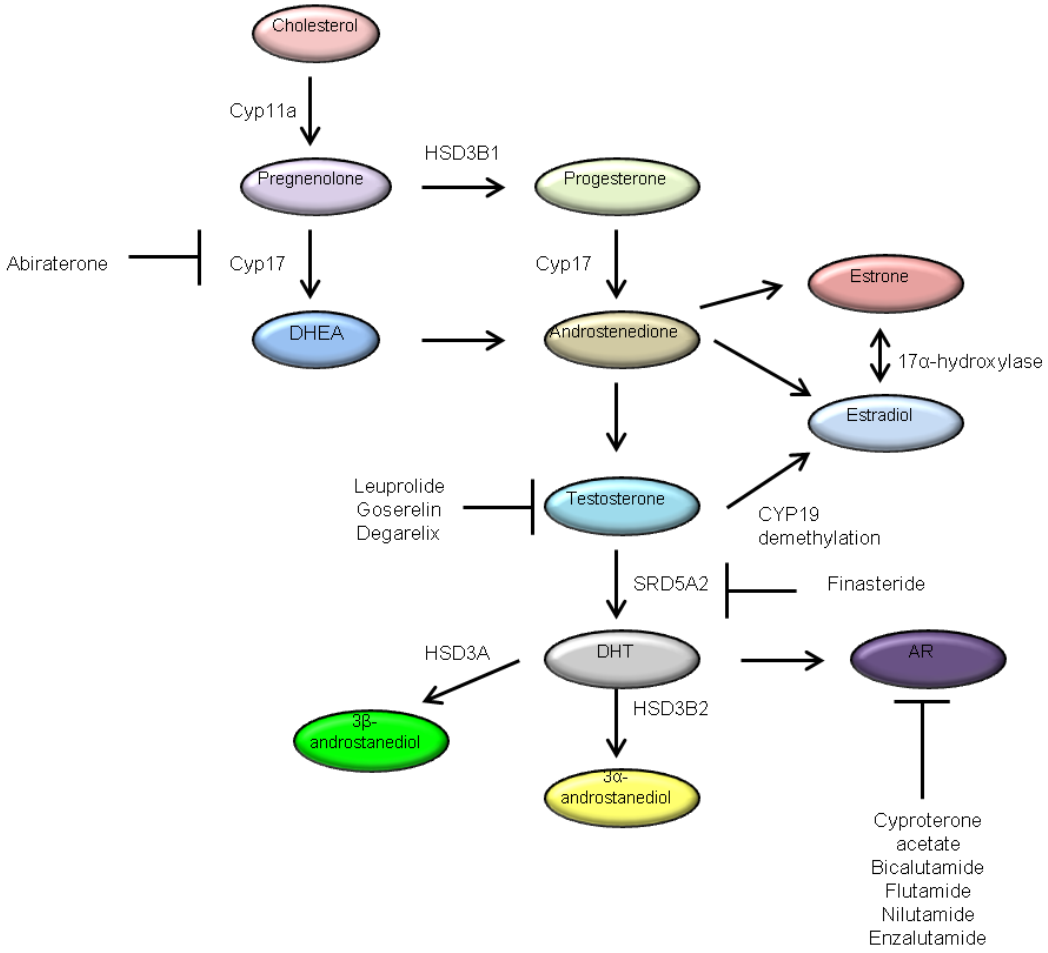


Figure 14 Schematic representation of the Steroid biosynthesis pathway and drugs that target the pathway and inhibit AR function

Testosterone is converted to DHT via SRD5A2 and binds to AR to activate AR-dependent transcription. Drugs that prevent the action of AR include cyproterone acetate, bicalutamide, flutamide, nilutamide and enzalutamide. As well as testosterone, the pituitary gland, adrenal androgen precursors pregnenolone and progesterone can activate testosterone through androstenedione. Abiraterone targets the Cyp17 enzymes responsible for the production of DHEA and androstenedione.

1.4.4.5 Combined androgen blockade.

Medical castration has been shown to lower testosterone levels by 95%. However, circulating androgens from the adrenal glands, dehydroepiandrosterone (DHEA) and androstenedione still remain, and continue to be converted to androgens in PCa cells (Labrie et al. 1993). Combined androgen blockade (CAB) works by combining both castration and antiandrogen therapy so that not only are the majority of circulating androgens eliminated, but any remaining androgens of adrenal origin are prevented from binding to AR (Hellerstedt and Pienta 2002).

1.4.5 Castration resistant PCa

1.4.5.1 Chemotherapy

Docetaxel ($C_{43}H_{53}NO_{14}$) (Figure 15) has a molecular weight of 807.88 g/mol and functions by inhibiting mitosis through microtubule disruption. It achieves this by binding to free tubulin, promoting assembly of stable microtubules whilst inhibiting their disassembly. Docetaxel is a semi-synthetic taxane analogue initially approved by the FDA in 1996 for the treatment of advanced breast cancer (BCa), before demonstrating promising results in early phase trials for the treatment of CRPC (Picus and Schultz 1999). This led to a phase III study that showed a 24% reduction in the risk of death in a group of men treated with docetaxel in combination with prednisone in comparison to mitoxantrone in combination (Tannock et al. 2004). 45% of men in the docetaxel group also experienced a 50% reduction in serum PSA and improved quality of life including pain reduction. After an extended follow-up of this study 18.6% of patients survived ≥ 3 years in the docetaxel group in comparison to 13.5% in the mitoxantrone group (Berthold et al. 2008). Despite clinical advances made by treatment with docetaxel, PCa continues to progress, leading to a surge in the number of drugs approved for the treatment of mCRPC.

Cabazitaxel is a more recent second-line treatment for mCRPC. Cabazitaxel ($C_{45}H_{57}NO_{14}$) (Figure 15) has a molecular weight of 835.93238 g/mol and functions through microtubule inhibition. Initially, patients who progressed on docetaxel were given mitoxantrone, which produced only marginal PSA responses and no survival benefit. Cabazitaxel is a novel taxane that has proven efficacious *in vitro* and *in vivo* in docetaxel resistant cells (Galsky et al. 2010). It has been given as a second line chemotherapy to patients who progressed on docetaxel (Mottet et al. 2011). The TROPIC trial randomised 755 patients with mCRPC pre-treated with docetaxel and treated with either Cabazitaxel (25 mg/m^2) or mitoxantrone (12 mg/m^2), each in combination with a daily dose of prednisone (10 mg). Progression free survival was 2.8 months in the Cabazitaxel group compared to 1.4 months in the mitoxantrone group (de Bono et al. 2010). Despite improved quality of life and pain control with Cabazitaxel the National Institute for Clinical Evidence recommended against the routine use of Cabazitaxel for PCa treatment (NICE press release) in the UK, due to the price of the drug, weighed against the benefits. Despite the high number of drugs available for the treatment of castration

resistant PCa, each drug has limited activity and none are curative, leading to the need for more effective therapies.

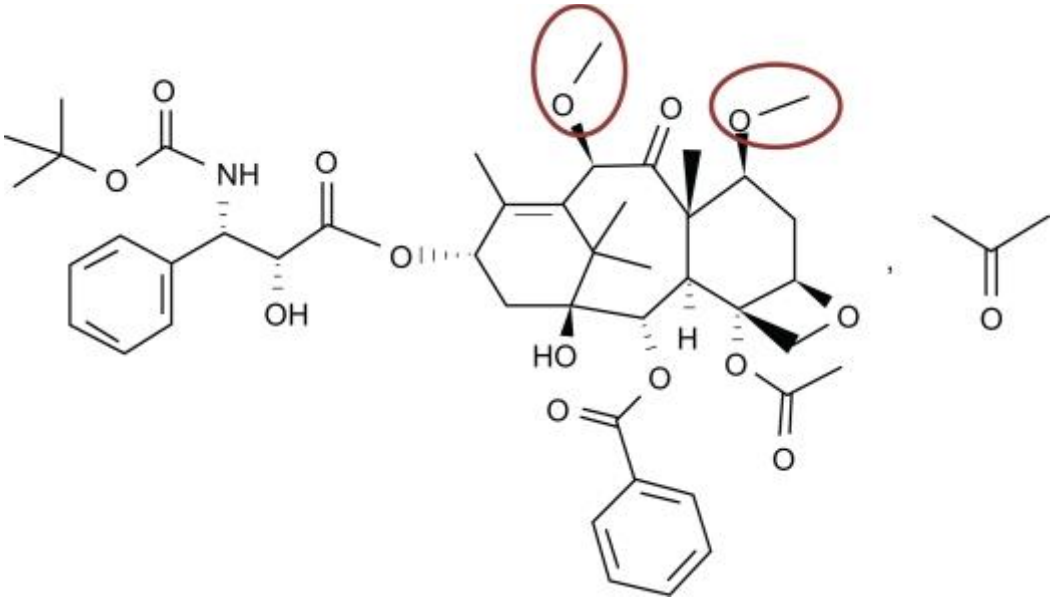


Figure 15 Schematic representation of the Docetaxel/Cabazitaxel

The schematic shows the structure of Docetaxel and the structural difference for Cabazitaxel. The red circles highlight the methoxy side chains that are substituted for the hydroxyl groups that are normally found in docetaxel.

1.5 Tissue specific Gene-directed Enzyme-prodrug therapy (GDEPT)

1.5.1 Gene-directed enzyme prodrug therapy

Gene directed enzyme prodrug therapy, also known as suicide gene therapy, was first proposed by Moolten over 25 years ago (Moolten 1986). It was developed as an alternative to chemotherapy as a way to solely target cancer cells, avoiding off-target side effects as a result. An ideal suicide gene therapy system must: 1) Use a prodrug that is non-toxic or minimally toxic to cells and highly toxic after activation, 2) The prodrug must be capable of entering the tumour and be taken up by the cells, 3) the drug must not be lost from the cells before the metabolite can kill the cells, therefore the metabolite half-life must be long enough to induce sufficient cell death, for example the half life of 5-Fluorocytosine (5-FC) in patients is 3-4 hours (Vermes, Guchelaar, and Dankert 2000) and, 4) the enzyme must be expressed specifically in the targeted cells in order to avoid off target toxicity. Cytosine deaminase (CD), cytosine deaminase uracil phosphoribosyl transferase (CD/UPRT) herpes simplex thymadine kinase (HSV-tk) and nitroreductase are four of the most well established enzymes utilized for enzyme prodrug therapy (Bourbeau et al. 2004, Koyama et al. 2000, Fogar et al. 2007, Ayala et al. 2006, Miles et al. 2001, Patel et al. 2009, Young et al. 2008).

HSV-1 encodes 70 genes, of which some are immediate early genes such as thymidine kinase (TK). TK forms a 376 long amino acid protein that is necessary when the virus enters the lytic cycle from a dormant stage. HSV-tk has a very broad substrate specificity including pyrimidines and pyrimidine analogs. HSV-tk converts ganciclovir (GCV), a purine analog, into a toxic metabolite as well as phosphorylating the nucleoside anaologs penciclovir and acyclovir. Once phosphorylated, these analogs are further phosphorylated by cellular kinases to their corresponding nucleoside triphosphates that mimic guanosine. These analogs are incorporated into nascent DNA and inhibit DNA synthesis, leading to a halt in replication and apoptosis (Kokoris and Black 2002).

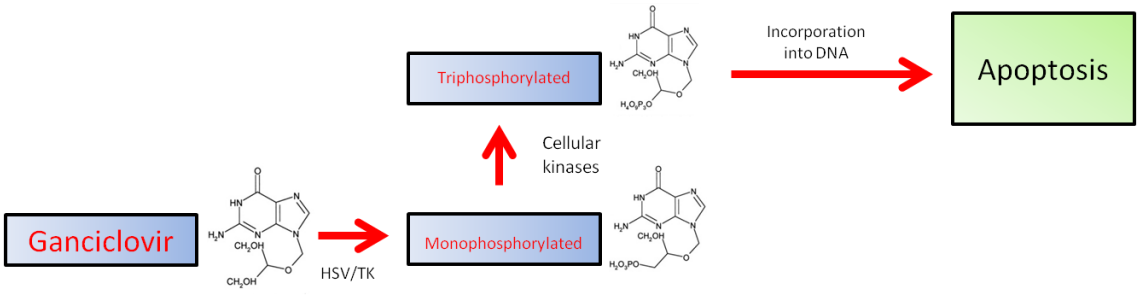


Figure 16 HSV/tk + Ganciclovir enzyme prodrug therapy

GSV, a purine analog is converted by HSV-tk into a toxic metabolite. GSV is further phosphorylated by cellular kinases to the corresponding nucleoside triphosphates that mimic guanoisine. This anolog incorporates into the nascent DNA, resulting in replication halt and apoptosis.

The first phase I clinical trial utilising HSV-tk for PCa therapy was conducted by Baylor college of medicine in 1999 (Herman et al. 1999). Treatment lead to increased PSA doubling time, a significantly increased reduction in mean PSA levels, and a significantly longer time to return to initial PSA levels after preliminary or repeat vector injections, as well as demonstrated safety of Ganciclovir gene therapy in men (Miles et al. 2001). Since this initial trial there have been several other phase I/II clinical trials incorporating HSV-tk that demonstrated safety after repeat cycles with limited efficacy (Shalev et al. 2000, Hassan et al. 2000, Teh et al. 2004).

Another well characterised prodrug activating enzyme is CD/UPRT (cytosine deaminase/ uracil phosphoribosyl transferase). The CD prodrug therapy system was initially created using bacterial CD. However, this gave a poor turnover rate of 5-FC by CD and was therefore replaced by the yeast CD (yCD). yCD displayed increased efficacy in CD/5-FC therapy and much greater kinetic properties towards 5-FC with a 22-fold lower km (Kievit et al. 1999). CD is a pyrimidine salvage enzyme that catalyses the deamination of cytosine to uracil. As a consequence of this, CD converts 5-FC to 5-Fluorouracil (5-FU) which is then converted to 5-FUMP by UPRT (Lundegaard and Jensen 1999) (Figure 17). UPRT is a member of the pyrimidine salvage pathway and is responsible for catalysing the synthesis of UMP or 5-FUMP from uracil or 5-FU. 5-FUMP is an active metabolite that inhibits RNA synthesis, DNA replication and thymidylate synthase (TS) activity (Longley, Harkin, and Johnston 2003), resulting in lethal DNA damage. It has been shown that some tumour cells display resistance to 5-FU due

to possible defects in downstream cellular metabolism or over expression of catabolic enzymes (Harris et al. 1994). Due to this, co-expression of CD with UPRT was tested and shown to enhance cell killing (Tiraby et al. 1998).

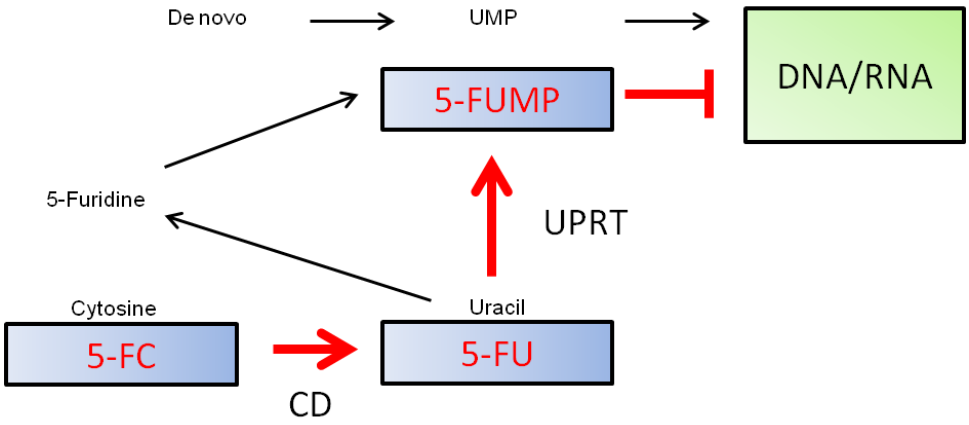


Figure 17 5-FC CD/UPRT enzyme prodrug system

CD catalyses the conversion of 5-FC to 5-FU. 5-FU is then further converted to 5-FUMP by UPRT, resulting in lethal DNA damage.

Finally, nitroreductase has been extensively used in a number of gene therapy vectors (Latham et al. 2000, Young et al. 2008), including a number of clinical trials, where is has proven its safety in a clinical setting in PCa and liver cancer (Palmer et al. 2004, Patel et al. 2009, Crack 2013).

CB1954 was found to be highly active *in vitro* against Walker rat 256 Carcinoma cells (Roberts, Friedlos, and Knox 1986). In this setting CB1954 was converted to a highly potent cytotoxic agent by endogenous rat DT-diaphorase (Knox et al. 1988), not present in humans, and therefore explaining why activity was poor in human tumours. Later studies demonstrated that the nitroreductase enzyme, encoded by the *nfsB* gene found in E.coli B, can convert CB1954 into its cytotoxic form 100-fold more efficiently than rat DT-diaphorase (Knox et al. 1992). Subsequently, numerous studies have investigated the clinical benefit to using nitroreductase and CB1954 in a gene-directed enzyme prodrug system and have proven efficacy and safety in combination with the CB1954 prodrug. Patel et al trialled a replication-deficient adenovirus (CTL102) encoding nitroreductase, driven by the CMV promoter, in combination with CB1954 in a phase I/II clinical trial and demonstrated safety and tolerability, as well as preliminary evidence of a change in PSA kinetics in patients (Patel et al. 2009). Furthermore, this system has worked particularly well in combination with granulocyte macrophage colony-

stimulating factor (GM-CSF), immunotherapy *in vitro* and phase I/II clinical trials are currently ongoing (Young et al. 2008, Crack 2013).

One advantage to using suicide gene therapy is the bystander effect; the ability of toxic metabolites to diffuse into and be taken up by surrounding cells. This allows less prodrug to be administered to the patient with enhanced levels of cell killing. Where toxic metaboliates are unable to diffuse into and be taken up by surrounding cells the bystander effect works through Gap junctions. These are narrow connecting channels between 2-3 nm in diameter that exist between cells. Gap junctions facilitate the exchange of small molecules including small metabolites, second messengers and electric signals through a procedure known as Gap Junctional Intercellular Communication (GJIC) (Goodenough and Paul 2009). Bystander activity of the 5-FU toxic metabolite has been previously demonstrated, though it has been revealed that unlike other drugs 5-FU does not require cell-cell contact to diffuse into neighbouring cells (Huber et al. 1994). Therefore, the mechanism of diffusion is not through intracellular gap junctions (Huber et al. 1994). 5-FU is a small molecule that is capable of diffusing into and out of cells; consequently this is thought to be the mechanism of spread for the toxic metabolite. However, studies have also demonstrated that the CD/UPRT fusion gene negatively influences bystander activity *in vivo* due to the fusion of UPRT and CD and therefore diminishes the therapeutic value of the CD/5-FC system (Johnson et al. 2011). This system will therefore suffer from reduced therapeutic window.

Suicide gene therapy needs to be targeted in order to avoid the off target side effects that are a result of chemotherapy. One approach is to utilize TSPs to drive enzyme expression and ensure that 5-FUMP is only produced in the cancer cells. For example if the *CD/UPRT* gene is placed downstream of a promoter containing androgen response elements, CD/UPRT will only be transcribed in cells that express functional AR. As AR is a key driver of PCa progression and survival, promoters that contain AR elements are suitable for use in targeted enzyme prodrug therapy (see section 1.6.2.4).

1.5.1 Utilising androgen responsive promoters for targeted therapy of PCa

Extensive studies have been performed that exploit the AR-dependent transcriptional activation of prostate specific promoters and enhancers for PCa

gene therapy, in order to increase safety and specificity of cytotoxic therapies, including prodrug converting enzymes, cytokines, siRNA and p53. A variety of prostate specific gene promoters have been investigated for this purpose including PSA, PSMA, Osteocalcin, human glandular kallikrein 2 (hK2) and the rat probasin promoter. Despite the discovery of thousands of novel Androgen regulated genes over the past decade, PSA still remains one of the most well characterised prostate specific genes (O'Keefe et al. 1998, Young et al. 1992, Balk, Ko, and Bubley 2003). PSA is currently used as a biomarker for the detection of PCa and is expressed in normal prostate epithelial cells and at all stages of prostate carcinogenesis, rendering it an ideal candidate for restricting gene expression to prostate cells. Previous studies have utilized the 6 kb regulatory region upstream of the PSA promoter that encompasses a TATA box, two AREs and upstream enhancer regions that have been shown to augment prostate specific expression (Cleutjens et al. 1997, Pang et al. 1997, Schuur et al. 1996). Within the entire 6 kb region a high affinity ARE was located (Cleutjens et al. 1996) together with four non-consensus AREs (Huang et al. 1999), consisting of 440 bp that collectively became known as the core PSA enhancer. Despite promising levels of activity from the PSA promoter and enhancer, this promoter was still inherently weak. Details of further PCa clinical trials that utilise androgen responsive promoters can be found in section 1.6.2.4, Table 9.

1.5.2 Increasing transgene expression levels from tissue specific promoters

In recent years many prostate-specific gene regulatory regions have been investigated for their specific activity, however they are inherently weak, resulting in low level transgene expression from these promoters. A number of methods have been investigated to enhance transgene expression from weak TSPs including; 1) Posttranscriptional enhancement of transgene levels (Choi et al. 1991), 2) creating chimeric promoters that are still capable of maintaining tissue specificity (Wu, MATHERLY, et al. 2001, Li et al. 1999, Latham et al. 2000), and finally 3) transcriptional amplification from a TSP (Iyer et al. 2001).

1.5.2.1 Chimeric promoters

Creating chimeric promoters has been one of the most popular methods of increasing transgene expression, often including the use of enhancers in combination with a tissue specific promoter (TSP). These promoters are aimed at

targeting the heterogeneous nature of PCa, utilising promoters that function in a number of different cancer types, whilst simultaneously increasing transgene expression.

To date the most effective PSA promoter has been chimeric, including a duplicated core enhancer together with the proximal promoter (Wu, Matherly, et al. 2001, Latham et al. 2000), that drove 20-fold higher expression than the PSA promoter alone (Wu, Matherly, et al. 2001). However there is still no effective therapy in clinical trials that utilises this chimeric promoter.

More recent chimeric promoters have included the incorporation of critical transcriptional elements from different prostate specific promoters. It was hypothesised that this would allow transgene expression in a much larger percentage of cells due to the heterogeneous nature of cancer, leading to increased efficacy. Perhaps the most elegant example was fusion of the regulatory enhancer regions of PSA and PSMA genes, resulting in high levels of luciferase expression in PSA and PSMA expressing PCa cell lines, irrespective of androgen expression (Lee et al. 2002). This chimeric enhancer, known as PSES, was later inserted into a conditionally replicating adenovirus, whereby viral replication was placed under the control of the PSES promoter (Li et al. 2005). Systemic administration resulted in the dramatic inhibition of CWR22rv tumours injected with AdE4PSESE1a, compared to tumours treated with a control virus. The PPT promoter, containing a T-cell receptor gamma-chain alternate reading frame (TARP), PSMA enhancer and PSA enhancer is the most complex chimeric promoter described to date and has also reported high level activity in androgen positive and negative cancers (Cheng et al. 2004). However, despite promising preliminary results with these novel promoter/enhancers they were still unable to drive sufficiently high levels of transgene expression.

1.5.2.2 Transcriptional amplification -the two-step transcriptional amplification system

A number of Two Step Transcriptional Amplification Systems (TSTA) have been developed that allow the amplification of a transgene from a TSP, while retaining target cell specificity. Transgene expression is amplified from a TSP via expression of the yeast transcriptional activator GAL4, fused to VP16, a strong transactivation domain from HSV-1 VP16. The GAL4-VP16 fusion protein binds to

multiple Gal4 binding sites upstream from the transgene, thereby amplifying transgene expression (luciferase in Figure 18), while retaining tissue selectivity.

Zhang.et.al modulated the activity of the TSTA system by: 1) placing potent PSA enhancers upstream of GAL4-VP16, 2) increasing the number of Gal4 binding sites from 1 to 5, this modification increased transgene expression with the PSA promoter/enhancer activator by 240-fold, 3) duplication of the VP domain, resulting in 2-fold increase in expression and 4) placing all the TSTA components on a single plasmid, rather than the traditional approach of keeping the gene specific promoter and reporter gene on separate plasmids. Overall, the modifications made drove 10-fold higher transgene expression than when using separate constructs (Zhang, Adams, et al. 2002). This construct was then gifted to Xie and colleagues for their work on pancreatic cancer.

Xie and colleagues proved the efficiency of the transcriptional amplification system in pancreatic cancer using the CCKAR promoter, that is overexpressed in pancreatic cancer (Weinberg et al. 1997). CCKAR is a G-protein coupled receptor, that upon binding by Cholecystokinin (CKK), takes part in regulating the secretion of enzymes and postprandial effects in the gall bladder (Takata et al. 2002). The promoter region is 724 bp long and has been identified upstream of the start site. It lacks the typical TATA and CAT binding sites for transcriptional regulation (Miller et al. 1995). The induction of the TSTA was able to increase the expression of luciferase from the original promoter present in the original construct by between 252 and 826-fold in three different pancreatic cancer cell lines, AsPC-1, PANC-1 and Panc02 (Xie et al. 2007). Xie and colleagues then replaced the luciferase gene with *BikDD*, a potent proapoptotic gene, and inhibited growth of Colo357FG and Colo357l3.6pl pancreatic tumours *in vivo* (p=<0.01) (Xie et al. 2007).

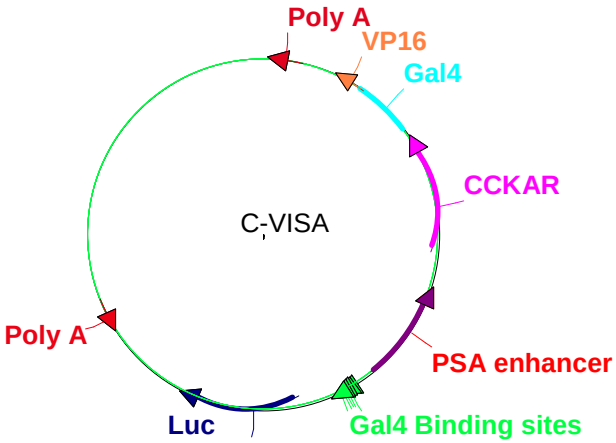


Figure 18 Plasmid map of CCKAR plasmid

Binding of CKK to the CCKAR promoter results in transcription of the GAL4/VP16 fusion protein. The fusion protein binds to 5 Gal4 binding sites upstream of the luciferase gene, resulting in increased transgene expression

1.6 Utilising Adenovirus for improved therapeutic transduction.

1.6.1 Adenovirus biology

1.6.1.1 Adenovirus classification

Adenoviruses were first identified in 1953, when Rowe and colleagues found pathogenic changes in primary cell cultures derived from adenoid tissues, which were a result of replication of a previously unidentified virus. This virus induced cytopathic changes in cultures of human cells (ROWE et al. 1953). However, it was not until 1954, when Hilleman and Werner isolated agents from respiratory secretions of army recruits, that the relationship between these viruses was established. They were later named adenoviruses after the original adenoid tissue in which the virus was discovered (HILLEMAN and WERNER 1954). The human adenovirus is part of the adenoviradea family, of which there are four genera: Mastadenovirus, Aviadenovirus, Atadenvirus and Siadenovirus. Mastadenovirus infect mammals and aviadenovirus infect birds, while the other two genera infect a broad range of hosts including reptiles and amphibians. Human adenoviruses belong to the Mastadenovirus genus and 53 different human adenovirus serotypes have been identified (Smith et al. 2010). These serotypes can be divided into seven species (A-G) based on their ability to agglutinate human erythrocytes and their biological, physiochemical and genetic properties (Table 6).

Table 6 Classification of Adenoviruses (Shenk 2001, Zhang and Bergelson 2005)

Species	Serotypes	Receptors	Tropism	Disease
A	12, 18, 31	CAR, FIX, FX	Cryptic (enteric and respiratory)	Mainly asymptomatic
B1	3, 7, 16, 21, 50	CD46, DSG-2, FX, CD80, CD86	Respiratory, ocular	Respiratory and ocular diseases
B2	11, 14, 34, 35	CD46, DSG-2, FX, CD80, CD86	Renal, ocular, respiratory	Respiratory diseases
C	1, 2, 5, 6	CAR, FIX,FX, Lf, DPPC, VCAM-1, HSPG, MHC1-α2	Respiratory, ocular, lymphoid	Respiratory and Ocular diseases
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 24-49,	SA, CD46, CAR, fx	Ocular (enteric)	Ocular diseases

	51			
E	4	CAR	Ocular, respiratory	Respiratory and ocular diseases
F	40, 41	CAR	Enteric	Gastroenteritis
G	52	ND	Enteric	Gastroenteritis

1.6.1.2 Adenovirus structure

The human adenovirus has a molecular weight of ~150 MDa. The adenoviridae are icosahedral particles with a diameter of ~950Å, containing non-enveloped viral dsDNA, 36 kb in size (13% of mass), encoding more than 40 different proteins (87% of the mass, 13 of which have been demonstrated to form the virus particle), no membrane or lipids and trace amounts of carbohydrate (Smith et al. 2010). The icosahedral morphology of the adenovirus is a product of the interactions of three primary capsid proteins (pII, pIII and pIV), and four minor proteins (pIIIa, pVI, pVIII and pIX) (Vellinga, Van der Heijdt, and Hoeben 2005) (Figure 19).

A virion consists of a protein shell (capsid) made of 252 subunits (capsomeres), of which 240 are homotrimeric hexons made from the major protein pII, and 12 are pentons (pIII). There are four hexons that are positioned in different areas of the capsid: H1, H2, H3 and H4 (Burnett 1985). H1 hexons are termed peripentonal hexons, of which 60 are associated with the pentons at the 12 apices. The remaining hexons are known as ‘groups of nine’ present on the 20 faces of the icosahedrons and further defined as H2 (on the two-fold axes) H3 (on the three-fold axes) and H4. There are hexon differences that are dependent on serotype including up to nine hyper variable regions situated on top of the molecule, 6 of which are resolved as α-helical rods in the 6 A˚ structure (Saban et al. 2006). A major part of the virus-neutralizing activity is as a result of at least one of these hypervariable regions.

The 12 pentons are located on each of the 12 apices and consist of the major capsid protein penton base at the base, with an extended trimeric fibre protein (pIV). The fibre protein is composed of three polypeptide pIV molecules, incorporating an N-terminal domain (attached to the penton through a non-covalent interaction), a central shaft and a C-terminal knob region. This is a major virus capsid protein and the first component to interact with target cell receptors and tissues. As well as association with a trimeric fibre protein, each penton is also associated with the minor capsid protein pIIIa, which stabilises the capsid through interaction with both hexons and pentons. The remainder of the four minor

capsid proteins, pVI and pVIII also work to stabilise the capsid. pVIII specifically stabilises the bond between peripentonal hexons and the rest of the capsid, while pVI and pIX stabilise the capsid through interaction of adjacent hexons and pentons.

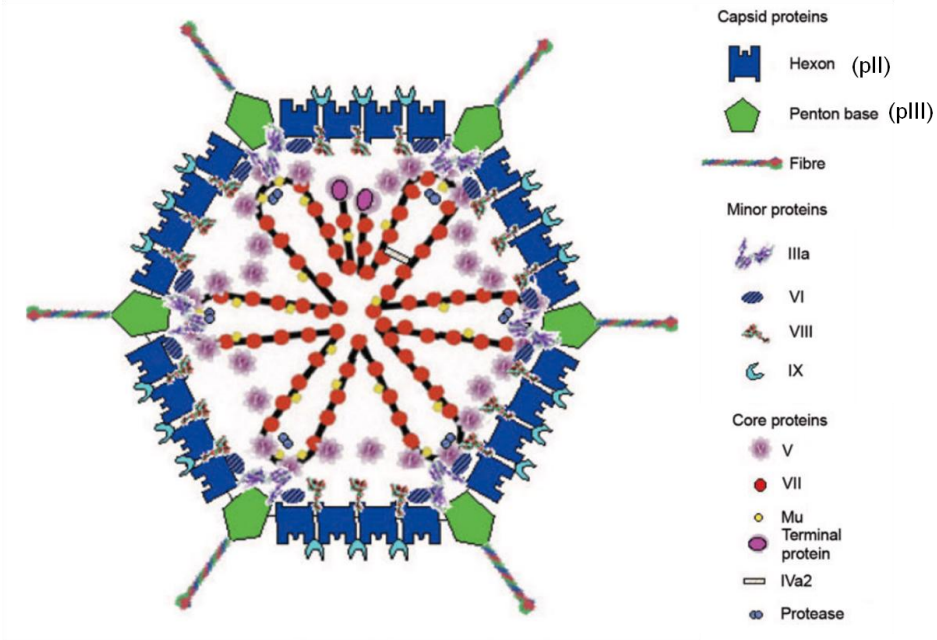


Figure 19 Adenovirus structure

Representation of adenovirus structure based on cryo-electron microscopy and crystallography. Image taken from (Russell 2009).

The remaining structural components; V, VII, Mu, Iva2 and terminal protein (TP) are associated with the dsDNA genome, constituting a 23K virion protease which is essential for assembly of the virion. There are about 160 copies of pV and very little is known about this polypeptide. However, evidence suggests that pV is directed to the nucleus and nucleolus in Ad5-infected cells and may aid the dissociation of core proteins, unveiling the viral DNA for replication/transcription. pV is also loosely associated with pVII and viral DNA (Harpst, Ennever, and Russell 1977, Russell, McIntosh, and Skehel 1971), with tighter interactions with polypeptide VI, of which there are 360 copies (Matthews and Russell 1998). Over 800 copies of polypeptide VII exist in each virion, spread along the length of virus DNA to which it binds strongly due to its highly basic nature (Russell and Precious 1982). pVII is thought to play a role in nuclear import of the viral DNA, targeting the genome to the nucleus and nucleolus. pVII has also been shown to restrict viral transcription at the early stages, but facilities late transcription and viral assembly at the end of the adenoviral life cycle, due to E1A transcription-

dependent release of pVII. Mu displays similar properties to protamines. Little is known about this core protein, however it can duplicate the nucleolar functions of pV and act as a precursor to modulate early expression of E2 proteins through its precursor (preMu), resulting in a shift in late protein expression. The core protein pIVa2, present in only a few copies, is critical for adenoviral packaging. It mediates encapsidation through interaction with pVII and L4 22K, a non-structural adenoviral protein, and binds to a specific stretch of the adenoviral DNA via repeat sequences. Finally terminal protein (TP) functions as a primer for DNA replication as well as binding of the viral genome to the nuclear matrix.

1.6.1.3 Genome organisation

Ad5 is one of the most well characterised adenovirus serotypes. The Ad5 genome was completely sequenced in 1992 and is 35935 bp in length. Adenoviral DNA is made up of inverted terminal repeat sequences that range from 40 to 160 bp, these inverted terminal repeats are responsible for circularization of single-stranded viral DNA through base-paring with their terminal sequences to produce panhandles, that are thought to be important for viral DNA replication (Shenk 2001). The adenovirus contains two identical origins for DNA replication present in each terminal repeat. Additionally, the viral genome also includes a cis-acting packaging sequence that directs interaction of the viral DNA with its encapsidating proteins, this sequence must be located within several hundred base pairs of the end of a chromosome to function correctly (Shenk 2001).

The adenoviral infectious cycle comprises of two phases, the early and late phases that occur before and after DNA replication respectively. The early phase, lasting around 6-8 h covers entry of the virus into the host cell, internalisation of the viral genome into the nucleus and transcription and translation of early genes. E1A, E1B, E2, E3 and E4 genes are responsible for this stage of the viral life cycle and the viral chromosome is subdivided into these early transcription units (Figure 20). IVa2 and IX genes are expressed at very high levels after the onset of DNA replication and are responsible for low basal levels of late transcription occurring at an early stage after adenoviral infection. The late phase of the infectious cycle takes around 4-6 h. The major late promoter is activated by IVa2 and IX gene products and is responsible for the primary transcription of late phase genes, which is subsequently split into five cassettes of transcripts termed L1-L5. This phase is responsible for the production of virus structural components and

maturation of the virus particle within the nucleus, including encapsidation of the particle. All viral genes are transcribed by RNA polymerase II, except for virus-associated (VA) I and II genes that are transcribed by RNA polymerase III (Shenk 2001). The functions of adenoviral proteins and their corresponding genes are summarised in Table 7.

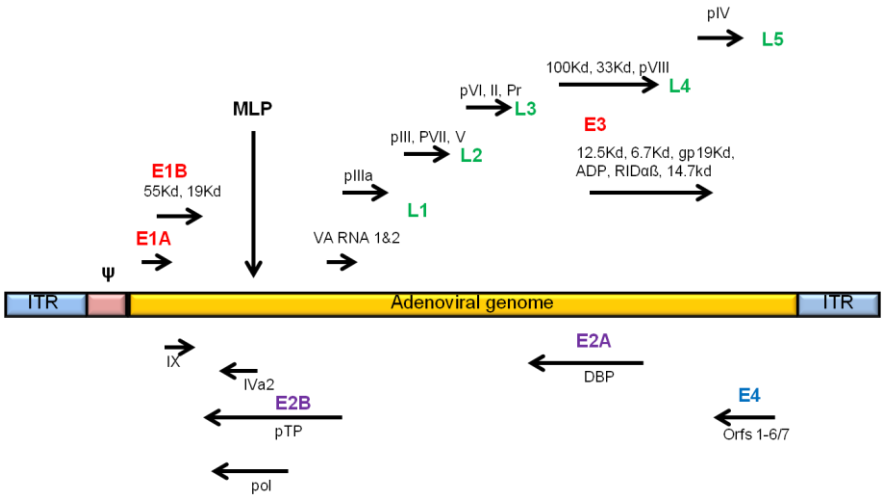


Figure 20 Schematic representation of adenovirus genome organisation

The early genes responsible for preparing the infected cell for replication and modulating immune response are shown in red (E1A, E1B and E3), whilst early genes involved in regulating viral DNA replication are shown in purple (E2A and E2B). The late set of early genes are shown in blue (E4). The late genes are transcribed from the major late promoter as one primary transcript that is subsequently processed, these are shown in green. Structural proteins are pII to pIX. Both strands of DNA are transcribed and arrows demonstrate the direction of transcription. The 103 bp inverted terminal repeats (ITRs) are located at the termini of the genome and are involved in viral DNA replication. The packaging signal (ψ) located from nucleotides 190 to 380 at the left end is involved in packaging of the genome into virion capsids. Adapted from (Russell 2000).

Table 7 Function of human Ad5 adenoviral particle proteins (Russell 2009, 2000, Gallimore and Turnell 2001, Wiethoff et al. 2005)

Gene	Protein	Function
E1A	E1A12S	Represses transactivating function of E1A13S through p300 binding as well as binding to many other cellular proteins including pRB (p107 &p130) & STAT1. p53 activator
	E1A13S	Essential for viral replication as it transactivates viral genes and binds to cellular proteins TBP, Sp1, CREB, DR1
E1B	E1B19Kd	Acts as an anti-apoptotic Bcl2 homolog
	E1B55Kd	Binds to p53 and represses transcriptional activity of p53. Works together with E4orf6 to stimulate late viral nuclear mRNA export, inhibits host cell mRNA export and degrades cellular proteins in an E3 ubiquitin ligase complex.
E2A	Polymerase	5'-3' polymerase activity and 3'to 5' exonuclease activity- viral polymerase
	DBP	DNA-binding protein Chain elongation for DNA replication

E2B	Pre-terminal protein	Core protein: essential for viral DNA replication, and circularisation of the genome
E3A	12.5Kd	Unknown
	6.7Kd	Unknown
	gp19Kd	Prevents presentation of viral antigens in infected cells by binding and sequestering MHC in the endoplasmic reticulum
	(ADP)	Adenovirus death protein -Promotes viral release
E3B	10.4/14.5Kd RID	Mediates internalisation of the FAS receptor and inhibits pro-apoptotic phospholipase A2
	14.7Kd	Interacts with caspase-8 in FLICE to inhibit FAS and TNF ligand-induced apoptosis as well as inhibiting pro-apoptotic phospholipase A2 and binding and blocking pro-apoptotic FIP proteins
E4	Orf 1	Facilitates transformation
	Orf 2	Unknown
	Orf 3	Interacts with E1B55K
	Orf 4	Inhibits E1A activation of E2F
	Orf 6	Interacts with E1B55K for late viral RNA export and shuts off cellular mRNA processing
	Orf 6/7	Transactivates E2A promoter by binding to E2F-binding sites
Delayed early gene	IX	Minor capsid protein: stabilizes viral capsid through association with hexons and pentons
	IVa2	Core protein: crucial to the packaging process- binds DNA and activates late viral gene transcription
L1	IIIa	Minor capsid protein: Stabilizes virion capsid through association with hexons and pentons
L2	III -penton	Major capsid protein: essential for virus internalisation and transport into endosomes through interaction of αvβ3/αvβ5 with RGD peptide on penton base.
	V	Core protein: provides bridge between the core and the capsid and facilitates final uncoating stages of capsid proteins
	VII	Core protein: spreads along the length of viral DNA and binds tightly due to highly basic nature, mediates localisation of virion to nucleus and diminishes early transcription
L3	II- hexon	Major capsid protein: Associates in trimers to form hexon capsomere-there are four kinds of hexons, (H1, H2, H3 and H4) and 240 hexons in an adenoviral capsid.
	Protease	Core protein: cleaves the precursors to the structural proteins IIIa, VI, VII, VIII, pTP and X. Essential for production of the infectious viral particle and uncoating during virion internalisation
	VI	Minor capsid protein: stabilizes virion capsid through hexon and penton interaction. Ruptures the membrane of early endosomes to mediate virion escape during infection
L4	100K	Associates with hexons during virion assembly and stimulates late viral translation
	VIII	Minor capsid protein: stabilizes virion through interaction with hexons and pentons providing a bond between peripentonal hexons and the rest of the capsid
L5	IV	Major capsid protein: associates in trimers to form penton, the first virus component to interact with a target receptors/tissues
VA RNA	VA1 &VA2	Prevents PKR-mediated protein synthesis shutoff and blocks α- and β- interferon anti-viral response as a result

Mu	Core protein: Similar properties to protamines. Modulates E2 gene expression to switch from early to late viral transcription and condenses chromatin during packaging.

1.6.1.4 Adenovirus life cycle

1.6.1.4.1 Virus attachment, internalisation and trafficking to the nucleus

Human adenovirus will primarily infect epithelial cells, even if other cell types can be infected, they are poor supporters of the adenoviral life cycle. Attachment of adenovirus subtype 5 is mediated in two-steps; firstly through the distal carboxy-terminal domain of the fiber protein. This domain terminates in a knob that binds to CAR (coxsackie adenovirus receptor) protein. CAR is a 46 kDa transmembrane cellular receptor belonging to the immunoglobulin superfamily, that serves as a high affinity receptor for all subtypes of adenovirus excluding B and G (Shenk 2001) (Table 6). Ad5 also interacts with a number of other cellular receptors to facilitate viral attachment *in vitro*, including; vascular adhesion molecule (VCAM-1), heparin sulphate proteoglycans (HSPG), and the major histocompatibility complex one alpha domain (MHC1-α2).

The second step in adenoviral attachment following CAR binding involves binding of the adenovirus penton protein to αvβ3/αvβ5 integrins, through an arginine-glycine-aspartic acid (RGD) motif, resulting in viral internalisation. This step is essential for most adenoviruses, with the exception of species F, which do not contain an RGD binding motif. This penton base-integrin interaction results in integrin clustering, stimulating activation of PI3K, MAPK and ERK downstream signalling pathways as a result. Induction of the PI3K/AKT pathway induces downstream events including the polymerization and reorganization of actin filaments for clathrin mediated endocytosis. The p38/MAPK pathway activation results in increased IL-8 production, suggestive of the host immune defence system and ERK1/2 activation, stimulating increased CAR clustering and activation of β-integrin subunits, whilst simultaneously up-regulating the p38/MAPK pathway (Coughlan et al. 2010, Russell 2000).

After successful attachment to cellular integrins by the RGD motif, internalization of the virion via clathrin-coated pits and subsequent endocytosis or macropinocytosis begins. Once internalised, the vesicles are acidified by the

vacuolar H+/ATPases, causing a change in pH to pH4.6-6.0, this is responsible for driving conformational changes to the structure of the virion, including the loss of capsomeres from the apices. The infecting particle must then exit the endosome and enter the cytosol, this process is mediated by the lytic action of pVI. After these processes are complete, 90% of the partially degraded virion successfully moves into the cytosol with a half time of 5 min, suggesting that the virus escapes the endosome prior to formation of a lysosome (Shenk 2001). Once in the cytosol, virus particles are transported across the cytoplasm through microtubules to the nucleus, via the microtubule associated motor dynein, which facilitates Ad attachment to microtubules. pVII is thought to be the primary viral mediator of this process. Hsc70 and nuclear histone H1 and H2 import factors, importin β and importin 7 are recruited by capsid interactions with CAN/Nup214. This facilitates complete capsid disassembly and delivery of viral genomic DNA to the nucleus (Coughlan et al. 2010).

1.6.1.4.2 Viral gene transcription

As previously discussed (section 1.6.1.3), viral gene transcription comprises a two-phase event that can be split into early and late phases. In order to induce adenoviral gene expression three main goals must be achieved. Firstly S phase of the cell cycle must be induced in the host cell, providing an optimal environment for viral replication. This stage utilises E1A, E1B and E4 gene products. Secondly, the infected cell must be protected from various host cell defence responses by expressing viral defence factors, including the E1B, E3, E4 and VA RNA genes that contribute to these defences. Finally, viral gene products required for viral gene replication must be synthesised. Accomplishment of all three of these goals is dependent on activation of the viral genome. The principal activating proteins of adenovirus are encoded by the E1A gene (Shenk 2001).

The E1A promoter is constitutively active and E1A is the first transcription unit to be expressed after the viral chromosome enters the nucleus. E1A encodes two mRNAs during the early phase of infection, with three additional E1A mRNA species, of no distinct function, accumulating at a later stage in the infectious cycle. The two early E1A mRNAs contain identical 5’ and 3’ ends with differing internal sequences that result in alternative splicing, encoding identical proteins, except for an additional 46 amino acid segment present in the larger polypeptide. These differential polypeptides are referred to as E1A12S (243 amino acids) and

E1A13S (289 amino acids) (Figure 21). The E1A proteins of different subtypes of human adenoviruses are comprised of three conserved regions, CR1, CR2 and CR3 that are separated by less highly conserved domains. These three domains play a large role in protein-protein interactions, facilitating the role that E1A12S and E1A13S play in binding to cellular proteins to modulate their function, rather than directly binding with the DNA. In fact, the E1A proteins bind to a variety of cellular transcription factors and regulatory proteins to activate transcription in three ways: 1) they mediate basal transcription through direct interaction with auxiliary factors, 2) they interact with activating proteins that bind to upstream promoter and enhancer elements and 3) they influence the activity of DNA binding factors by interaction with regulatory subunits (Shenk 2001). A list of E1A binding partners can be found in Table 8.

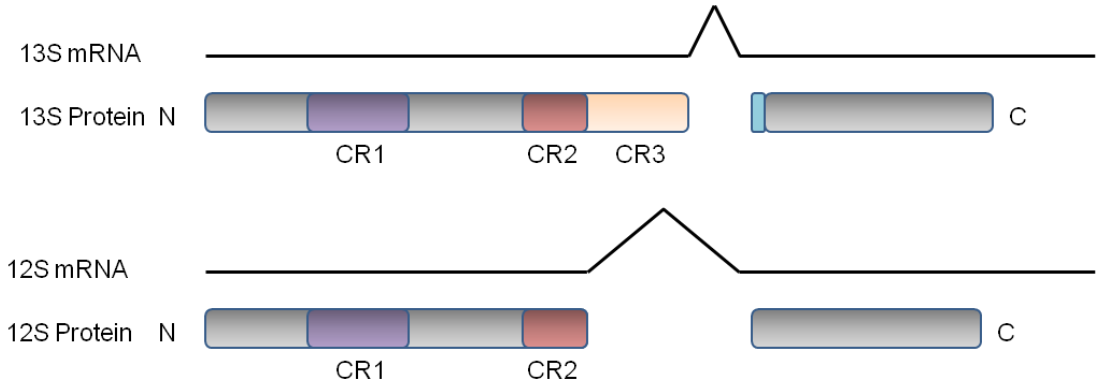


Figure 21 Diagram of E1A mRNAs and the polypeptides they encode

Diagrammatic showing the two different E1A mRNAs and the polypeptides they encode. Cellular proteins bind through the conserved regions (CRs) of E1A. E1A13S contains an additional CR3 domain, which functions as a strong activation domain.

E1A12S and E1A13S differ only in the presence of the CR3 domain in E1A13S, which functions as a strong activation domain through direct binding to the TATA-binding protein (TBP) as well as MED23, a subunit of the MED complex and CREB. TBP is the DNA-binding subunit of the auxiliary transcription factor IID (TFIID), which binds to the TATA box, an element present 25-30 bp upstream of transcription initiation sites in many genes. The cellular tumour suppressor protein p53 can also bind to TFIID. In fact, the p53 binding domain overlaps that of E1A13S, resulting in the displacement of p53 binding due to E1A binding and a relief in p53 mediated transcriptional repression. E1A12S can also activate transcription through the TATA box, but in a more indirect way, the 12S and 13S proteins can both bind directly to Dr1, a factor that normally binds to TBP, and

inhibits transcription. The 12S and 13S proteins sequester Dr1 through direct binding preventing Dr1 interaction with TBP.

E1A proteins can also activate transcription through E2-F binding sites. E2-F forms a complex with the cellular retinoblastoma tumour suppressor protein pRB, which in turn inhibits transcriptional activation by E2F. Both E1A proteins bind through CR1 and CR2 to pRB, releasing E2F and driving transcriptional activation as a result. This E2F activation not only affects the expression of viral genes, but also accelerates cell cycle progression through upregulation of cellular genes, including genes important for S phase and cell growth (Shenk 2001). Despite evidence pointing towards the absolute requirement for E1A in adenoviral gene transcription, E1A-deleted mutants have been shown to replicate in some cell types. Dl312, a virus lacking the E1A gene, replicated at a similar rate to wild-type virus in HeLa cells that were infected at a high MOI, but not in numerous other cell types. E2F and E4F sites have also been identified in E2 and E4 promoter regions, suggesting that cellular transcription factors could bind to other early viral promoters in the absence of E1A, stimulating low levels of transcriptional initiation (Shenk 2001).

Table 8 Major E1A-binding partners

E1A-binding protein	Binds 12S E1A	Binds 13S E1A
pRB	+	+
p107	+	+
p130	+	+
p300	+	+
TBP	-	+
hTAF	-	+
SUR-2	-	+
Dr1	+	+
ATF-2	-	+
YY1	+	+
Sp1	?	+
MAZ	?	+
STAT1	+	+

1.6.1.4.3 Induction of S-phase in the host cell

Adenovirus infection can induce quiescent cells to enter the S phase of the cell cycle. Protein products of the early adenoviral genes create the optimal cell environment for viral DNA replication, through interaction with cellular proteins including pRB pocket proteins and the p300/CBP proteins. This modulation is

mediated by CR2 and the non-conserved amino-terminal domains of the E1A proteins.

E1A-binding through the CR2 domain to pRB is primarily responsible for entry into S phase. Over expression of pRB inhibits cell cycle progression through E2F binding, causing cells to arrest in middle to late G1 phase. The activity of pRB is regulated by cyclin-dependent kinases and hyperphosphorylation of pRB prevents the formation of the cell cycle inhibitory pRB-E2F complex. The LxCxG motif sequence present in the CR2 domain binds to pRB. The E1A-binding domain on pRB, referred to as the binding pocket, also binds E2F. Therefore, E2F can be released from pRB by E1A, leaving E2F free to activate cellular genes that promote cell cycle progression (Berk 2005). Similarly, CR1 also weakly interacts with pRB, playing an auxiliary role in stabilising the CR2-pRB interaction (Shenk 2001). E1A can also interfere with the function of pRB independent of competitive binding. pRB functions as a transcriptional repressor through interaction with HDAC containing complexes and hBRM and BRG1 members of the SWI/SNF transcriptional regulators to promoter complexes, resulting in cell cycle arrest. Thus, the binding of E1A to pRB disrupts these interactions, consequently inhibiting transcriptional repression (Gallimore and Turnell 2001).

E1A can also stimulate cell cycle progression through an alternative pathway involving p300/CBP. These proteins bind to the poorly conserved region in CR1 and are therefore able to stimulate DNA synthesis in viral mutants that lack the CR2 domain, essential for pRB dependent cell cycle progression. More recently, p300/CBP have also been demonstrated to bind to the CR3 domain (Pelka et al. 2009). p300/CBP proteins exhibit intrinsic histone acetyltransferase activity and recruit other HAT proteins including PCAF, as well as acting as coactivators of a number of transcription factors that include CREB, STATs and nuclear receptors. Consequently, binding of p300/CBP to E1A displaces and inhibits the intrinsic acetyltransferase activity of PCAF. Thus, S-phase entry is facilitated by the ability of E1A to inhibit the function of p300/CBP. p300/CBP also serves as a coactivator of p53, whose loss of function contributes towards tumour progression. Consequently, E1A proteins antagonise p53 function through inhibition of p300/CBP activity, resulting in the unblocking of cell cycle progression. This process is not however enough to stimulate progression into S phase (Pelka et al. 2009).

1.6.1.4.4 Viral genome replication

Accumulation of E2 gene products is required for viral DNA replication. This takes place between 5-8 h after initial infection of the cell, when the products of the early genes have established optimum conditions for DNA synthesis and continues until the host cell dies.

Viral replication takes place in two stages, in the first stage only one of the two DNA strands acts as a template for DNA synthesis, displacing the other strand of DNA and producing a duplex that consists of a daughter and parental strand. The complement to the displaced strand is then synthesised in the second stage. This process requires circularisation of the second strand, through the self-complementary termini, resulting in a duplex termed a panhandle. This panhandle allows recognition of the replication machinery to initiate DNA synthesis, resulting in completed duplexes that consist of one parental and one daughter strand (Figure 22). In order for this process to take place the ITRs located at both ends of the viral DNA act as replication origins comprised of cis-acting sequences. Three functional domains have been defined within the 51 bp terminus of the ITRs, termed A, B and C. The first 18 bp of viral DNA is termed domain A, functioning as a minimal origin of replication with base pairs 9 to 18 being fully conserved among all serotypes of human adenovirus, that recruits viral encoded pTP and DNA polymerase. Domain B (base pairs 9-39) and domain C (base pairs 40 to 51) bind cellular factors including nuclear factor 1 (NF1) and NFIII respectively. pTP binds covalently to the 5' end of the viral chromosome and acts as a primer for DNA replication, whilst preserving the integrity of the viral chromosomes ITR sequence throughout multiple rounds of replication. pTP also interacts with CAD, a multifunctional pyrimidine biosynthesis enzyme within the nuclear matrix that helps to anchor the adenoviral replication complexes close to useful cellular factors. pTP and polymerase bind together as a complex and the polymerase utilises both 5'-to-3' polymerase activity as well as 3'-to-5' exonuclease proof-reading activity. Chain elongation requires both DNA-binding protein (DBP) and NFII. In the presence of DBP, the polymerase is highly processive, travelling the entire length of the chromosome after separation from pTP. Furthermore, NFII is necessary to overcome DNA structural problems that arise after extensive replication and has been identified as having topoisomerase activity (Shenk 2001).

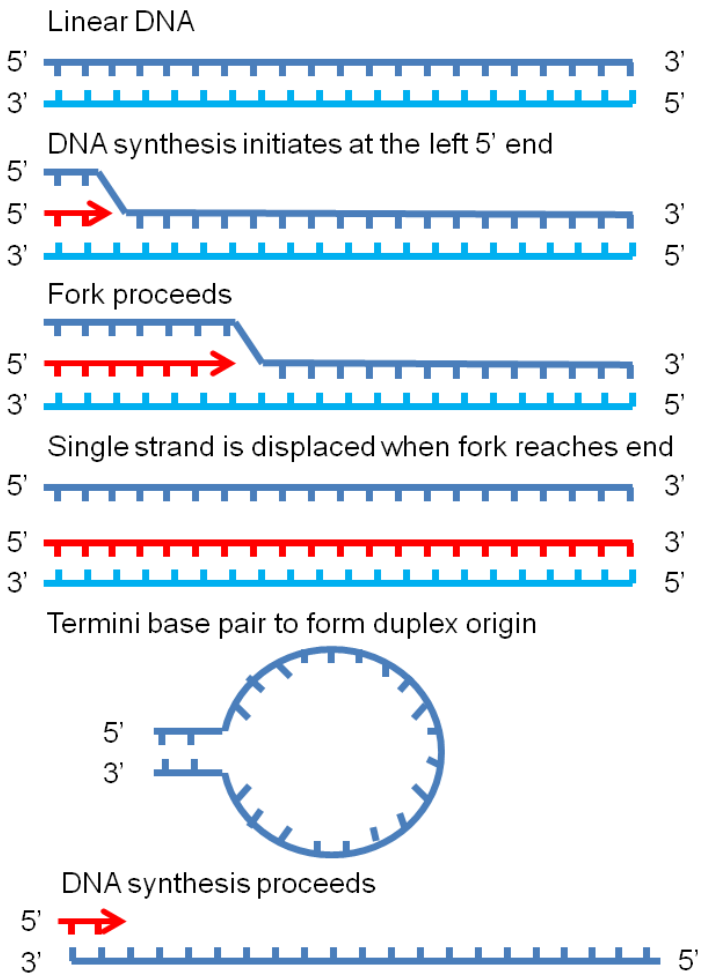


Figure 22 Schematic representing adenoviral replication

DNA synthesis is initiated at the 5' end of the DNA, as the DNA replication fork proceeds it displaces the other strand, leaving a newly synthesised double stranded DNA and the displaced single strand. The single strand circularises through its self complimentary termini, forming a panhandle, allowing for DNA synthesis to commence from the 5' end, resulting in production of completed duplexes that consists of one parental and one daughter strand.

1.6.1.4.5 Viral assembly and release

Upon the onset of DNA replication adenoviral late gene expression begins. During replication of adenoviral DNA, large quantities of adenovirus structural polypeptides are produced that enable viral assembly. The late coding regions are organised into a single transcript and subsequent post transcriptional modifications result in 5 different mRNA transcripts termed L1 to L5. Accordingly, these transcripts encode components essential for viral maturation and encapsidation and their expression is controlled by the major late promoter (MLP). The MLP exhibits low level activity in the early stages after infection, becoming

several hundred-fold more active after DNA replication. This increased expression is due to binding of the USF/MLTF transcription factor that binds upstream of the MLP after the completion of DNA replication. Additionally, the product of the delayed early gene Iva2 also binds to the MLP and contributes to its induction through interaction with USF/MLTF (Shenk 2001).

Upon the initiation of DNA replication and the synthesis of all late mRNAs, accumulation of cellular mRNAs in the cytoplasm is halted. This process is mediated by the E1B55kD and E433kD polypeptides that exist in a complex, facilitating shutdown of host protein synthesis. L4-100K fulfils this function by preventing phosphorylation of eIF-4F, an initiation factor that binds to the cap of cellular mRNAs and facilitates scanning of the 40S ribosome from the cap to the AUG. The five late viral mRNA families encoded by the late transcription unit are uncapped and contain a tripartite leader sequence, an identical sequence of 200 nucleotides in the 5' non-coding region that is important for mRNA translation in the later stages of infection. This sequence is responsible for the preferential translation of adenoviral late mRNAs and continued translation well after infection, due to a process known as 'ribosome jumping', in which the 40S ribosome can scan from the cap, to AUG without the requirement of the eIF-4F helicase (Shenk 2001).

Viral DNA replication and accumulation of structural proteins initiates viral assembly. Assembly of the hexon trimers requires L4 100-kD, which acts as a scaffold to facilitate assembly. Experiments have indicated that the penton base and fibre assemble independently, subsequently joining to form the penton capsomere. Next, the hexon and penton capsomeres assemble in the nucleus, where virion assembly is initiated. Viral DNA enters the capsid either coupled to encapsidation while DNA replication is taking place, or independent of encapsidation, in separate nuclear compartments. It is unclear as to which mechanism is correct. It has been established that the viral DNA enters the empty capsid via the packaging sequence that allows for DNA-capsid recognition. pIVa2 subsequently binds to the viral DNA, together with L152.55Kd and pVII to initialise encapsidation. The L3-coded proteinase then cleaves the pVI, pVII, pVIII and pTP to stabilize the viral particle and render it infectious (Shenk 2001).

There are two systems in which a virus will escape and spread. Firstly, the intermediate filaments that form the cytoskeleton are disrupted. For this purpose

the L3 proteinase cleaves the cellular cytokeratins k18, preventing polymerization, a process that is required to form filaments. This process compromises the integrity of the cell, making the infected cell more susceptible to lysis. The second system involves the E311.6Kd protein, otherwise known as the adenovirus death protein (ADP). This protein kills as it accumulates in late stage infection and facilitates release of the progeny virions (Shenk 2001). The mechanism by which this process occurs is unclear, however studies utilising mutated ADP have shown loss of localisation to the nuclear envelope and Golgi, correlating with regions important for N- and O- linked glycosylation. These processes result in lower protein stability and reduced lytic behaviour (Tollefson et al. 2003). A figure summarising the adenoviral life cycle can be found below (Figure 23).

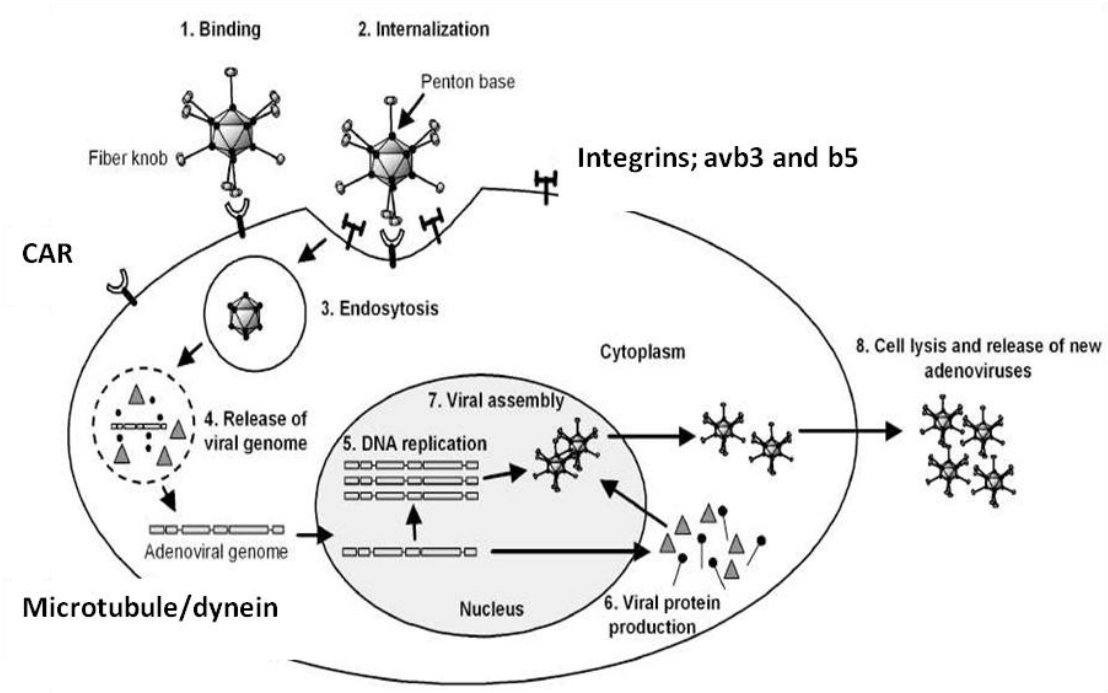


Figure 23 Summary of Human adenovirus life cycle

Adenovirus binds through the fiber knob to CAR receptors expressed on the epithelial cell surface. The penton base then binds with the integrin $\alpha_v\beta_3$ and the virus is internalised. The virus is partially disassembled and the capsid travels to the nucleus through the microtubule network. Once in the nucleus viral gene transcription is initiated, resulting in progeny formation, eventually causing cell lysis and resulting in the release of new adenoviruses.

1.6.2 Viral gene therapy

In the year 2000 the first genetically linked disease, X-linked severe combined immunodeficiency (SCID-X1), was cured through gene therapy treatment. A total of 20 infants were treated across three countries. From a long-term follow up of the French trial in 2010, that initially consisted of nine children, eight patients were still alive between the ages of 8 and 11 (Hacein-Bey-Abina et al. 2010). Unfortunately, 30 months post-treatment one of the 9 patients treated in the French trial developed T-cell leukaemia, as a direct result of the integration of the retroviral vector used for therapy near the LMO2 proto-oncogene. This insertion led to aberrant transcription and expression of LMO2 and disease progression (Hacein-Bey-Abina, Von Kalle, et al. 2003). Following this unfortunate turn of events, a further three patients from the French arm of the trial and one English patient also developed T cell leukaemia. Subsequently, 4 out of 5 patients were successfully treated for Leukaemia and remain in complete remission. This turn of events lead to the trial being placed on hold while efforts were made to improve the safety of viral vectors used for gene therapy. Almost a decade after the initial retroviral gene therapy trial, a vector with improved safety has been utilized for clinical trials and has demonstrably retained its efficacy for the treatment of SCID-X1, whilst demonstrating significantly less insertion site clustering within LMO2 and MECOM. However, the long term effects on leukemogenesis with this new vector are still unknown (Hacein-Bey-Abina et al. 2014). This first example of utilising gene therapy for the treatment of disease not only highlighted the importance of developing a safe vector for transgene introduction, but demonstrated the real potential for the role of viral gene therapy in the treatment of disease and cancer. Identification and modification of viral vectors that demonstrate improved safety, while maintaining clinical efficacy, is therefore one of the most fundamental aspects to developing an effective gene therapy strategy.

1.6.2.1 Oncolytic and replication-defective adenoviruses

Adenovirus can be used as a classical gene therapy tool for delivering therapeutic genes (replication defective viruses), as a drug alone through lysing cancer cells and even as a combination of both (oncolytic adenoviruses). Due to higher transfection efficiencies viral vectors are most commonly used for gene directed enzyme prodrug therapies. Replication defective adenoviruses contain a deletion of the E1A gene, responsible for viral replication, but can also include deletions of

the E3 genes, generating ~6 kb space for the insertion of therapeutic transgenes. Replication defective adenoviral vectors provide higher levels of safety than oncolytic viruses, but decreased transduction efficiency leading to poor efficacy in the majority of cases. For this reason replication defective adenoviruses are often administered in combination with therapeutic drugs that synergistically increase target cell death. Alternatively, oncolytic adenoviruses benefit from increased transduction and have also demonstrated safety in numerous clinical trials. However, replicating viruses can only take up to an additional 10% of the viral genome (~3.6 kb), reducing the capacity for the insertion of therapeutic transgenes. This becomes problematic when large expression cassettes include two-step-transcriptional amplification systems as well as therapeutic transgenes, as there is not sufficient space to allow for the incorporation of the whole cassette. Thus, it is important to establish which method will increase efficacy and transduction whilst remaining clinically safe.

1.6.2.2 Oncolytic gene therapy as an anticancer agent

In the beginning of the last century, several observations were made that natural viral infections were associated with cancer remission. Since then, adapting Human adenoviral vectors (Adv) for use as anti-cancer treatments has been a major focus in the application of viral gene therapy due to: 1) adenoviral vectors have generated a good safety profile through numerous clinical trials 2) they can easily be produced under good manufacturing practice (GMP) conditions 3) batches can be purchased that meet high safety standards 4) wild-type Adv vectors are only mildly pathogenic 5) they are easily manipulated by genetic modifications 6) they can be engineered to include new polypeptide ligands in their capsids that are able to preferentially infect specific cell or tissue types. 7) They can be modified to carry therapeutic or reporter genes, including prodrug activating enzymes (de Vrij et al. 2010). 8) They do not incorporate into cellular DNA in contrast to the viruses described in section 1.6.2.

To this day Adenoviruses remain the most popular vectors for viral gene and immunotherapy evaluated in clinical trials, with a total of 488 adenoviral vectors tested at various phases (www.abedia.com/wiley). This accounts for 22.8% of all gene therapy clinical trials, but is closely followed by Retrovirus (19.1%) and naked/ plasmid DNA (17.7%). Of the 488 gene therapy clinical trials utilising adenovirus, 351 are targeted to cancer and are at various clinical phases. There

have been 53 clinical trials using Adenovirus to treat PCa (www.abedia.com/wiley).

Other methods to selectively target cancer cells include engineering viruses to complement mutations found only in cancer cells. These are normally associated with cell cycle and apoptosis. These viruses are known as replication selective, as they take advantage of deregulated pathways in cancer cells. Replication selective viruses become replication competent in cancer cells, where loss of function mutations are dispensable for replication, whilst remaining inactive in normal cells. One of the most well studied examples of this is the ONYX-015 virus (Bischoff et al. 1996). This virus has a deletion in the E1B55k gene, which ordinarily represses p53-mediated transactivation and works together with E4orf6 to promote p53 degradation and prevent premature cell death. This virus therefore complements cancer cells with a non-functional p53, either through direct p53 mutation, or mutation in both upstream and downstream regulators of p53. Consequently, ONYX-015 will selectively replicate in these cells as the E1B deletion renders the virus unable to degrade p53 during viral replication, resulting in ineffective replication in normal, p53 expressing cells. Despite promising initial trials utilising ONYX-015, further investigations showed that the virus was unable to replicate efficiently in some p53-defficient cell lines, or some cell lines harbouring p53-related mutations (Goodrum and Ornelles 1997, Edwards et al. 2002). More worryingly ONYX-015 was also found to replicate in p53-expressing normal cells and cancer cells that retained wildtype p53 (Goodrum and Ornelles 1997, Rothmann et al. 1998).

Notwithstanding the varied *in vitro* data generated with ONYX-015, 18 Phase I and II trials have been conducted to date. ONYX-015 has been explored both as a monotherapy and in combination with chemotherapeutics. Administration of ONYX-015 as a single agent failed to generate efficacy, with less than 15% of cases resulting in a clinical benefit to patients with head and neck cancer (Nemunaitis et al. 2000). Despite some promising trial results many patients still responded poorly to ONYX-015 and the clinical effect was varied. The poor responses were due to a number of factors including attenuation of immune defence due to the E3B deletion and the virus being unable to support viral nuclear mRNA export. Adaptations of ONYX-015 that improved efficacy have therefore been investigated, including H101. Various clinical trials have confirmed the oncolytic anti-cancer effect of H101, with a response rate of 30.4% in one

phase II trial combined with chemotherapy (Lu et al. 2004) and a 78.8% response rate for the treatment of head and neck cancer in combination with cisplatin and 5-FU, compared to a 39.6% response for cisplatin and 5-FU alone (Xia et al. 2004). These results led to the approval of H101 for cancer treatment in combination with cisplatin and 5-FU by the Chinese government in 2005. Efficacy was reported in treated tumours, however the patients were not followed up long-term (Garber 2006).

The poor efficacy of ONYX-015 as a monotherapy identified areas for improvement in the generation of novel oncolytic adenoviruses to improve efficacy. Increased efficacy generated with the H101 virus in combination with chemotherapy also highlighted areas in which combinatorial therapies could increase efficacy, whilst reducing toxic side effects associated with chemotherapeutics. The following strategies to engineer cancer selective oncolytic mutants have since been explored: 1) arming adenoviruses with therapeutic transgenes, 2) utilising tissue specific promoter to restrict adenoviral replication and 3) retargeting adenovirus attachment and infection.

In recent years the E1ACR2 and E1B19K oncolytic adenoviral mutants have demonstrated high selectivity and efficacy in combination with chemotherapeutics. These viruses retain functions essential for the viral life cycle by deletion of smaller gene regions (Oberg et al. 2010). Various E1ACR2 deleted mutants have demonstrated high efficacy in preclinical studies (Lamfers et al. 2002, Raki et al. 2008, Suzuki et al. 2002). E1ACR2 deleted mutants have also been tried in clinical phase 1 trials for Glioma and Ovarian cancer to establish the maximum tolerated doses of the viruses (Glioma Ad∆24RGD-4C (DNX-2401) NCT00805376, MD Andersen, Ovarian completed Ad24RGD NCT00562003 (https://clinicaltrials.gov). Double deleted mutants (Ad∆∆), with a deleted E1ACR2 and E1B19K region have been used in combination with cytotoxic drugs docetaxel and mitoxantrone to enhance cell killing in a number of PCa cell lines (Oberg et al. 2010). In fact, the double deleted virus was able to inhibit DU145 and PC3 tumour xenograft growth in combination with docetaxel, suggesting a potential clinical treatment for solid tumours in combination with chemotherapeutics (Oberg et al. 2010). Additionally, Ad∆∆ was combined with a number of phytochemicals that have previously proven anticancer activities. The combination of Ad∆∆ with equol and resveratrol synergistically increased cancer cell killing in PC-3 cells and tumour xenografts,

suggesting a safer alternative to more traditional combinatorial therapies with chemotherapeutics (Adam et al. 2012).

1.6.2.3 Challenges for AdV as a therapeutic vector

1.6.2.3.1 Immune system response to oncolytic viral infection

Although initial trials using adenoviral vectors have accumulated interesting results, they appear to be ineffective as monotherapies; there are still many hurdles that are necessary to overcome, including the innate immunity and increasing viral uptake into target cells. The adenovirus enters the cell by binding to CAR and integrins on the cell surface; however these are only expressed in very small quantities. Therefore, applications that specifically target adenoviral therapies to cancer cells, and as a result increase the concentration of virus entering the target cancer cells are desired. Most adenoviral therapies are injected directly into the tumour, and therefore interference from the immune system is not a major issue for the initial treatment. However, many gene therapy trials require repeat administration, risking host immune interference. Additionally, direct injection into the tumour does not target metastatic cancer cells, thus, more effective ways to evade the host immune system are required.

The innate immunity provides immediate protection against infection and is governed by type 1 interferons. Once they bind their receptors they trigger the transcription of many gene products called interferon stimulated genes (ISGs). As tumours evolve they become non-responsive to interferons and lose expression of key ISGs such as MHC genes, the protein products of which are required for antigen presentation to immune system cells. The tumour therefore becomes invisible to the host immune system (Parato et al. 2005).

After initial activation of the innate immune system the adaptive immune system is triggered by free circulating virion-associated or cell-associated gene products. Some viruses for example measles and HSV retain their therapeutic activity despite a large neutralizing antibody response, it is not yet known whether these viruses could be effectively delivered to disseminated tumours if injected intravenously. Using certain viruses including vaccinia, measles and poliovirus can harbour problems as many patients will already have a mature adaptive immune response of antibodies to the virus due to previous vaccination programs.

However, studies have shown that by administering naturally occurring mutants that have a propensity to form Extracellular enveloped viruses (EEV) it might be possible to evade pre-existing immunity, increasing tumour infectivity and spread of the oncolytic viral vector.

Another option is to mask the therapeutic virus from antibody neutralisation with chemical conjugates. Coating adenovirus with multivalent co-polymers renders the virus resistant to antibodies (Fisher et al. 2001, Green et al. 2004). This process has been shown to prevent complement activation and reduce the uptake of the virus by liver cells during systemic administration, resulting in a 10 fold increase in circulating virus. The only problem with this method of masking the virus is that the virus loses its infectivity as its cell attachment protein is conjugated. Therefore, a second targeting molecule must be linked to the polymer to facilitate viral infection (Parato et al. 2005).

1.6.2.3.2 Ad5 binding to human erythrocytes

Recent advances in research into adenoviral vectors have shown that Ad5 binds to human erythrocytes due to the presence of the coxsackievirus adenovirus receptor and the complement factor C4BP on the cell surface (Carlisle et al. 2009). It is thought this may have occurred through evolution to protect the body from systemic infection. Unfortunately, this binding dramatically reduces extravasation and infectivity, allowing erythrocytes to work as circulating 'virus traps'. Carlisle and colleagues showed that excess Ad5 fiber protein or anti-CAR antibody inhibits binding of Ad5 to human erythrocytes (Carlisle et al. 2009). CAR is the primary binding interaction between Ad5 and erythrocytes in PBS but complementation is also involved in the binding. Interestingly, the binding of erythrocytes to Ad5 only takes place in humans, not in other animals. Carlisle and colleagues therefore showed that the circulation time of Ad5 increases when washed human erythrocytes were injected into mice together with Ad5. The increased levels of Ad5 were predominantly associated with blood cells. The sequestration of Adv prevents the use of adenoviral vectors to treat metastatic PCa. If injected intravenously, the high affinity binding of virus to erythrocytes lower the amount of virus that would reach the tumour.

1.6.2.3.3 Adenovirus sequestration in the liver

After intravenous administration, adenovirus is predominantly sequestered by the Kupffer cells in the liver within minutes, resulting in hepatocyte uptake and liver toxicity (Shayakhmetov et al. 2004). This hepatocyte uptake poses a massive problem in generating viral efficacy after intravenous injection. Ad5 primarily binds and attaches to host cells through CAR and the fiber knob, followed by binding to cellular integrins (as discussed previously section 1.6.4.1). However, elimination of binding to both CAR and integrins in several studies have failed to abolish adenoviral liver transduction (Martin et al. 2003, Smith et al. 2002), suggesting an alternative pathway for Ad5 infection of liver cells. Understanding the mechanism by which adenovirus is cleared from systemic circulation is therefore crucial in the construction of more efficacious, targeted vectors.

1.6.2.4 Optimising adenoviral gene therapy through tumour selectivity

Wildtype adenoviruses target most epithelial cells as well as cancer cells, requiring viruses to be engineered to ensure tumour selectivity. As mentioned previously (see section 1.5.2), one method to ensure tumour selectivity is through the use of TSPs. Prostate specific promoter/enhancer regions have been widely used to restrict transgene expression in several clinical trials (Table 9). These TSPs promoters can be placed either upstream of the E1A gene in order to restrict E1A expression and viral replication to the prostate, or any other cell type, or in place of the E1A gene to control transgene expression in a replication deficient virus. Examples of this include the CG7060 adenovirus in which the PSA promoter/enhancer was inserted between the E1A promoter and the E1A coding region, resulting in replication in prostate tissues with elevated PSA levels. Clinical trials demonstrated the safety of this virus and evidence of decreased PSA levels was seen in patients with locally recurrent PCa (DeWeese et al. 2001)

Similarly, Yu et al showed efficacy *in vitro* utilising an adenovirus with the E1B gene expression controlled by the hK2 promoter, closely related to PSA and E1A gene expression by the PSA enhancer. This virus was significantly attenuated in non-prostate tissue resulting in a high therapeutic index (Yu, Sakamoto, and Henderson 1999). However, PSA promoter viruses were evaluated clinically with good safety but were unable to drive sufficient cell death *in vivo* due to their weak promoter activity. Steps were therefore taken to increase the activity of the PSA

promoter and enhancer, whilst continuing to restrict gene expression/viral replication to the prostate. The most effective method to achieve this so far has been to duplicate the core enhancer in combination with the promoter, responsible for achieving nearly 20-fold higher activity in comparison to the single enhancer construct. After successful insertion of this promoter/enhancer element into an adenovirus driving luciferase, strong prostate specific expression was demonstrated in comparison to the CMV promoter (Wu, Matherly, et al. 2001). Most prostate-specific promoters that have been investigated are regulated by the AR, however the osteocalcin (OC) and PSMA promoters have also been utilised in targeted therapies. OC is expressed in osteoblasts as well as primary and metastatic PCa cells. Koeneman et al demonstrated that OC driven HSV/tk therapy in combination with ganciclovir was effective in destroying PCa cell lines *in vitro* and tumour xenografts *in vivo* (Koeneman et al. 2000). This was later tested in a phase I/II clinical trial in combination with valacyclovir for the treatment of six men with androgen-independent PCa. All patients tolerated the therapy with no serious adverse side effects. However, only one PSA response from 318.3 to 4.9 ng/ml was observed and three of the patients who were docetaxel-naive had to receive chemotherapy after PSA levels progressed (Shirakawa et al. 2007).

PSMA has also been investigated as a novel biomarker for cancer progression and is highly expressed in poorly differentiated adenocarcinomas and metastatic cancer (O'Keefe, Su et al. 1998). Furthermore, PSMA is upregulated during androgen-deprivation, suggesting that its regulatory regions could be used as a TSP to target androgen-independent PCa (O'Keefe et al. 2000, Uchida et al. 2001).

Table 9 Prostate promoter/enhancers utilised for adenoviral gene therapy.

Regulatory element	Results summary	Ref
	Original promoters	
hK2	▪ E1a expression under the control of hk2 showed expression in PSA+ cells and prostate tumour selective replication	(Yu, Sakamoto, and Henderson 1999)
OC	▪ HSV-TK driven by OC promoter went to phase I clinical trials ▪ E1a and E1b expression controlled by OC promoter- replicated in OC-expressing cells and inhibited tumour growth *in vivo*	(Koeneman et al. 2000)
	Improved promoters	
Rat Probasin	▪ E1a placed under the control of Rat probasin selective replication in PSA+ cells ▪ Apoptotic gene Bas expressed specifically in prostate cells causing cell death *in vitro* and reduction in tumour volume *in vivo* ▪ Rat probasin specific expression of HSV/tk, growth suppression of AI tumours	(Yu et al. 1999, Zhang, Yu, et al. 2002, Furuhata et al. 2003)
PSE (PSA promoter/enhancer)	▪ Nitroreductase gene expression placed under the control of PSE was detected in PSA+ cells and was inducible by androgens, comparable expression to CMV	(Latham et al. 2000)
	Chimeric promoters	
PSMA/PSES	▪ E1A gene expression controlled by PSMA/PSES, replication restricted to prostatic AI cells and cell killing of PSMA+ cells *in vitro* and *in vivo* ▪ E1A and E4 expression controlled by PSMA/PSES drove prostate specific cell killing *in vitro* and *in vivo*	(Lee et al. 2004, Li et al. 2005)
PPT (PSMA enhancer/PSA enhancer/T cell receptor gamma chain)	▪ Used to drive a luciferase reporter, demonstrated high prostate specificity and high expression levels with and without testosterone *in vitro* and *in vivo*. Higher activity than CMV promoter following I.V administration	(Cheng et al. 2004)

1.6.2.4.1 Arming adenoviruses with therapeutic transgenes

1.6.2.4.1.1 Tumour suppressor protein p53

Introduction of a therapeutic tumour suppressor gene or abrogation of an oncogene that specifically targets tumour cells is an attractive prospect for gene therapy. p53 is the most widely used tumour suppressor gene in viral gene therapy, as it is one of the most commonly mutated genes in cancer. In fact, p53 mutations have been identified in 50% of human cancers with a total of 25,000 mutations currently reported (Chen et al. 2014). Ordinarily p53 induces cell cycle arrest, senescence and apoptosis in response to DNA damage and other stress signals. Mutation of p53 therefore plays an important role in malignant cell progression, suggesting restoration of wild type p53 may be an effective antitumor therapy. Several studies utilising adenoviral vectors to introduce p53 as a therapy have demonstrated dramatic cell death via apoptosis and cell cycle arrest in human glioma and glioblastoma cells *in vitro* and inhibited tumour growth *in vivo*

(Gomez-Manzano et al. 1996, Gomez-Manzano et al. 1997, Köck et al. 1996). The first marketable p53 based adenoviral vector was named Gendicine and was approved by the food and drug administration (FDA) in China on 16th October 2003 for head and neck squamous cell carcinoma treatment. Gendicine is a replication deficient Ad5 virus where the E1 region has been replaced by wild-type p53, linked with a Rous sarcoma virus (RSV) promoter. Gendicine functions by triggering apoptotic pathways, activating immune responses, inhibiting DNA repair and blocking survival signals (Chen et al. 2014). Gendicine has been clinically proven in a number of clinical trials with limited side effects, including fever. Evidence also suggests benefits of p53 mediated therapy include mobilising the host's immune system. However, low transduction rate of p53 from a replication deficient viral vector limits the effectiveness of this therapy. Replicating viruses were therefore developed using cancer-related gene promoters to enhance E1-dependent viral replication and induce higher p53 expression as a consequence. The adenovirus E1B55K protein binds to and blocks p53 function, preventing p53-induced cell death and apoptosis (Wiman 2007), and promotes viral replication as a result. One of the best and well studied of these replicating viruses is ONYX-015 (discussed previously section 1.6.2.2). ONYX-015 has the E1B55K gene deleted and can therefore only replicate in cells with a deregulated (inactivated) p53 pathway. Numerous clinical trials have demonstrated the low efficacy of ONYX-015 for the treatment of numerous advanced cancers, including head and neck cancer. Poor efficacy of this virus for use as a single agent and varied response rates from patients led to the development and licensing of the novel H101 virus (described previously section 1.6.2.2).

Despite proven efficacy and approval of both Gendicine and H101 these viruses are still not widely used due to poor efficacy and transport to metastatic sites. Ways to improve efficacy and delivery to metastatic sites must be explored to improve the clinical impact of these viruses on PCa treatment.

1.6.2.4.2 Re-targeting and de-targeting of adenoviruses

As mentioned previously (section 1.6.1.4.1), CAR plays a major role in virus cell attachment and internalisation. However, adenoviral vectors are inefficient at infecting cells *in vivo* due to neutralisation by pre-existing antibodies. A growing body of evidence has suggested that CAR may not be the primary receptor *in vivo*.

Type C adenoviruses have been shown to infect cells that do not express CAR, or express it in very low levels in the airway, liver and lymphocytes (Arnberg 2009). This suggests that receptors additional to CAR are responsible for adenoviral infection. Therefore, genetically engineering adenoviruses to modify their cellular receptors offers the opportunity to retarget adenovirus based on cell type specific receptors. Two approaches have been used to achieve this: 1) adapter based targeting strategies and 2) genetically modifying the capsid (Beatty and Curiel 2012).

The adapter based strategy utilises a molecular bridge to retarget the adenovirus from its primary receptor to a different cell surface receptor. The most common location to adapt the Ad virion is the fiber knob domain, as this allows retargeting of vectors, whilst de-targeting adenovirus from its primary CAR receptor. Conversely, genetic modification of the adenovirus capsid to retarget ligands has been widely investigated.

1.6.2.4.2.1 Small RNAs

More recently, studies have taken advantage of the gene silencing mechanisms of microRNAs (miRNAs) to control viral replication. miRNAs are small non-coding RNA molecules, typically 20-24 bp in length that target and bind specific mRNA sequences. Binding results in the catalytic degradation of the target mRNA. This method has been utilised to attenuate viral potency to normal tissue, e.g. the liver, while wild-type efficacy can be maintained at the tumour sites that do not express the relevant miRNA. This was demonstrated when four miRNA122 binding sites were inserted into the 3' UTR of E1A driving luciferase, inclusion of these sites caused an 80-fold decrease in luciferase expression in hepatocytes after intravenous injection in comparison to wild-type virus (Cawood et al. 2009). Leja et.al showed similar results by producing AdCgA-E1A-miR122, an miR122 targeted oncolytic adenovirus, where E1A gene expression fused to luciferase was placed under the control of the chromogranin A (CgA) promoter, with 6 or 12 repeats of miR122 binding sites in the 3' UTR of luciferase. CgA is highly expressed in neuroendocrine tumours and is used as a sensitive tumour marker. Lower E1A expression in murine normal hepatocytes was demonstrated following intravenous injection with AdCgA-E1A-miR122 than with wildtype virus. Repeated injection of AdCGA-E1A control virus, lacking the miR122 repeats induced liver toxicity in mice, whereas AdCgA-E1A-miR122 injection did not have the same

effect. This suggests that a combination of a specific transcriptional promoter with a post-transcriptional miRNA target will allow higher doses of virus to be administered, whilst avoiding off-target liver toxicity (Leja et al. 2010). More recently this method has been applied to replication incompetent adenoviruses where four repeats of miRNA122 binding sites were inserted into the 3' UTR of the E2A, E4 or pIX genes. Non-specific expression of these genes was suppressed from 2 to 100-fold compared with wildtype virus. In particular, the vector carrying miRNA122 binding sites targeted to the E4 gene suppressed E4 gene expression in the liver, resulting in significantly reduced hepatotoxicity (Shimizu et al. 2014). This adds to mounting evidence for the use of tissue-specific miRNAs to inhibit unspecific expression of viral genes, reducing off-target side effects as a result.

1.6.2.4.3 Genetic modification of the adenoviral capsid

1.6.2.4.3.1 Chimeric adenoviruses

An alternative method to re-targeting adenoviral infection involves genetically modifying the adenovirus virion to bind different ligands. It is essential to ensure that genetic engineering of the viral capsid does not interfere with capsid assembly. Thus, efforts have focussed on genetic manipulation of the fiber domain, the primary determinant of Ad infection. As previously mentioned, CAR is the primary receptor to most adenovirus serotypes excluding serotype B (Table 6). Studies have therefore focussed on utilising serotypes with alternative target cell receptors, for example CD46 in B serotype adenoviruses. The entire fiber of Ad5 is genetically replaced with its structural counterpart from a different adenoviral serotype and has shown great efficacy in a number of Ad5 resistant cancer types including ovarian (Rein et al. 2011) and prostate (Murakami et al. 2010). These viruses can include shaft and knob domains from different adenovirus species, for example the knob domains of Adv-3, Adv-11 or Adv-35 together with Adv-5 (Murakami et al. 2010). Chimeric adenoviruses have even advanced to incorporate nonhuman Ad serotypes, however the majority of targets for these vectors are still undetermined.

1.6.2.4.3.2 Peptide targeted adenoviruses

Studies of the adenoviral fiber knob domain have highlighted two separate locations that can be genetically engineered to present different cellular peptides

without disruption of fiber function. The first of the two regions is the C-terminus. Insertion of an integrin binding RGD motif into this area has previously yielded positive results *in vitro* and *in vivo* (Wickham et al. 1997). The second region that can be genetically engineered is the HI loop. This domain is an exposed loop structure connecting β-sheets H and I in the Ad knob domain that can handle peptide insertions of up to 100 amino acids without affecting the knob domain. Dimitriev et al. Inserted an integrin-binding RGD motif into the HI loop (Dmitriev et al. 1998), this virus enhanced efficacy and gene delivery in ovarian cancer cell lines (Murugesan et al. 2007). Several groups have also inserted cell-specific peptides into the HI loop that are highly specific, to target a range of cancers including Hsp47 targeted therapy for head and neck cancer (Li et al. 2008) and EGFR targeted therapy for glioma (Piao et al. 2009)

1.6.2.5 Adenoviral GDEPT clinical trials for PCa

Over the past decade gene therapy clinical trials have accelerated from the use of replication defective adenoviruses that contain a single therapeutic gene, to replication-competent oncolytic adenoviruses lacking a therapeutic gene, and most recently to oncolytic adenoviruses that contain multiple therapeutic genes. These therapies have been trialled as single agents and in combination with radiotherapy for the treatment of localized PCa. Replication-competent viruses have replaced replication deficient viruses due to their cytolytic activity, demonstrable specific anti-tumour activity and safety, high copy number replication, resulting in higher transgene expression, and a greater ability to spread only within the tumour.

These trials have also included the use of replication-selective adenoviruses for PCa gene therapy, relying on intraprostatic delivery of the virus in combination with 5-FC/GCV and intensity-modulated radiotherapy. The most effective therapies have included expression of both CD and mutTK in a replication-competent Adenovirus, whereby CD and mutTK expression is under the control of the constitutively active CMV promoter. These transgenes were inserted in the E1 region and the adenoviral death protein was present in the E3 region. (Freytag et al. 2007, Barton et al. 2006), that in one case have demonstrated transgene expression for up to 5 months (Barton et al. 2008). Similarly, encouraging results were obtained in a Phase I trial that utilized the wild-type HSV-1-TK gene (Herman et al. 1999). Only one in 18 patients (6%) suffered from specific treatment related

toxicity, potentially attributable to leakage of the virus into the systemic circulation. Moreover, PSA levels decreased by 50% in 17% of patients, lasting more than a year in one case.

A number of gene therapy trials have used adenoviral vectors expressing HSV-tk in combination with ganciclovir for high risk localised and locally recurrent PCa (Nasu et al. 2007). Two phase I/II trials replaced the E1 gene with restricted expression of HSV-tk using the Osteocalcin promoter, a major bone matrix protein expressed prevalently in PCa epithelial cells, for the treatment of metastatic PCa (Kubo et al. 2003, Shirakawa et al. 2007), but were not efficacious enough for further studies. However, despite increasing evidence of safety and efficacy, these viruses have never moved past phase III clinical trials. A summary of some of the adenoviral gene therapy clinical trials for PCa can be seen in Table 10.

Whilst hundreds of adenoviral gene therapy systems have been clinically tested, only Gendicine and H101 are licensed cancer treatments. However, these viruses are still not used worldwide for standard treatment. Although adenoviral vectors still remain the most promising and widely used gene therapy platform, problems still lie with generating efficacy. Steps to improve this, including the use of TSP's, targeting oncolytic viruses to cancer cells thorough modifications to viral proteins and improving transduction through altering surface molecules of adenovirus receptors have all enhanced the efficacy of many gene therapy strategies. Nonetheless, further modifications need to be performed in order to generate a vector with enhanced transduction that retains its cancer cell specificity, in order to produce an effective gene therapy system for cancer.

Table 10 Recorded adenoviral gene therapy clinical trials for PCa (http://www.abedia.com/wiley/).

Phase	Trial description	Result	Admin	Transgene	Ref
I	Adenovirus-mediated suicide gene therapy for PCa	No virus related cytopathic effect could be observed. Dose-dependent infiltration of T and B lymphocytes in the whole prostate and in tumour areas was observed. Boosting of adenovirus-specific antibody responses was observed in 7 patients, and an increased adenovirus-specific PBMC proliferation and IFN-gamma production was seen after Adv-HSV-tk stimulation.	I.T	HSV-tk	(van der Linden et al. 2005)
I	Trial of Adenoviral-Mediated Herpes Simplex Thymidine Kinase Gene Transduction in Conjunction with Ganciclovir Therapy as Neo-adjuvant Treatment for Patients with Clinically Localized (Stage T1c and T2b&c) PCa Prior to Radical Prostatectomy	Produced limited toxicity and evidence of antitumor activity following injection of the prostate. Furthermore, this system has been shown to direct systemic antitumor activity in several experimental cancer models, including that of PCa, which may serve as the basis for in-situ immunomodulatory gene therapy. In a mouse model of PCa, natural killer (NK) cells have been identified as the mediator of anti-metastatic activity following Ad-HSV-tk + GCV, resulting in the combination of Ad-HSV-tk and adenovirus-mediated expression of interleukin 12 (Ad.IL-12)	I.T	HSV-tk	(Hassan et al. 2000)
I	AdUP: A replication defective type 5 adenovirus vector expressing nitroreductase and GMCSF (AdNRGM) followed by intravenous CB1954, in patients with locally relapsed hormone-refractory PCa	Ongoing- initiated in 2013 the gene therapy is based on the intraprostatic injection of a viral vector (AdNRGM) carrying the GMCSF gene, which is able to induce a strong immune response against the PCa, and nitroreductase which is able to CB1954 (prodrug) to a powerful anti-cancer drug.	I.T	NTR/GMCSF	(Crack 2013)
I	Ad-OC-TK Plus Valacyclovir for the Treatment of Metastatic or Recurrent PCa	One prostate-specific antigen (PSA) response (from 318.3 to 4.9 ng/ml) was observed with a time to PSA progression (TTP) of 12 months. Docetaxel (30 mg/m^2 per week) and estramustine (560 mg/day). Combination chemotherapy (DE) was given to three docetaxel-naive patients on PSA failure after gene therapy. All three patients had a PSA response to DE therapy with 21, 7, and 4 months of TTP. These results suggest that additional trials are warranted.	I.T	TK/VAL	(Shirakawa et al. 2007)
I	Combined Suicide Gene Therapy and Radiation Therapy for Locally Advanced Carcinoma of the Prostate.	The results demonstrate that replication-competent adenovirus-mediated double-suicide gene therapy can be combined safely with conventional-dose 3D-CRT in patients with intermediate- to high-risk PCa.	I.T	CD/HSV- TK	(Freytag et al. 2003)
I	E1B-Attenuated Replication Competent Adenovirus Vector containing the E. coli Cytosine Deaminase/HSV-1 Thymidine Kinase Fusion Gene in Conjunction with Two Prodrugs, 5-Fluorocytosine and Ganciclovir for Patients with Local Recurrence of PCa after Radiation Therapy	Seven of 16 (44%) patients demonstrated a >or=25% decrease in serum prostate-specific antigen, and 3 of 16 (19%) patients demonstrated a >or=50% decrease in serum prostate-specific antigen. Transgene expression and tumour destruction at the injection site were confirmed by sextant needle biopsy of the prostate at 2 weeks. Two patients were negative for adenocarcinoma at 1 year follow-up.	I.T	CD/HSV- TK	(Freytag et al. 2002)
I	CV706 Replication-competent, adenovirus lacking therapeutic gene, E1A gene driven by PSA promoter, no E3 region genes	Short term—low toxicity, no DLTs, no MTD up to 1 × 1013 vp; encouraging PSA responses, five objective (25%) responses with one lasting 11 mo. Possible dose effect	I.T	-	(DeWeese et al. 2001)
I	Study of Adenoviral Vector Delivery of the HSV-tk Gene and the Intravenous Administration of Ganciclovir in Men with Local Recurrence of PCa after Radiation Therapy	Three patients achieved an objective response, one each at the three highest dose levels, documented by a fall in serum PSA levels by 50% or more, sustained for 6 weeks to 1 year. This study is the first to demonstrate the safety of ADV/HSV-tk plus GCV gene therapy in human PCa and the first to demonstrate anticancer activity of gene therapy in patients with PCa.	I.T	Hsv-tk	(Herman et al. 1999)
I	RTVP-1 gene therapy for PCa prior to radical prostatectomy	Toxicities included urinary tract infection, flu-like syndrome, fever, dysuria and photophobia. No pathologic complete remission was seen. Morphologic cytotoxic activity, induction of apoptosis and nuclear p27^{Kip1} upregulation was observed. Preliminary evidence suggests anti-tumour activity and systemic immune response.	I.T	RTVP-1	(Sonpavde et al. 2011)
I	Ad5-CMV-NIS therapy for locally recurrent PCa that did not respond to external-beam radiation therapy	The study is currently ongoing in human trials- experiments in canines demonstrated no vector-related toxicity and evidence of safety and efficacy.	I.T	NIS	(Dwyer et al. 2005)

I/II	Gene therapy in patients with PCa	Study ongoing Previous studies have demonstrated suppressed tumour growth in mouse models of prostate, breast and testicular cancer. Ad-REIC up-regulates systemic anti-cancer immunity.	I.T	REIC/Dkk-3	(Edamura et al. 2007)
I/II	Gene directed enzyme prodrug therapy for the treatment of PCa	No correlation between pre-existing immunity or the magnitude of the immune response to vector and the clinical outcome as measured by changes in serum prostate-specific antigen (PSA) level. Increased frequency of T cells recognizing prostate-specific antigens PSA or prostate-specific membrane antigen (PSMA) was detected in 3 of 11 patients after therapy, suggesting that this direct cytotoxic strategy can also stimulate tumour-specific immunity.	I.T	NTR + cb1954	(Onion et al. 2009) (Patel et al. 2009)
I/II	Study Evaluating HSV-tk + Valacyclovir Gene Therapy in Combination with Radiotherapy for PCa	Biopsy data was encouraging and appeared to show no evidence of malignancy earlier than historical data. Combined RT, short-course hormonal therapy, and in situ therapy appeared to provide good locoregional control but inadequate systemic control in patients with positive pelvic lymph nodes.	I.T	HSV/TK	(Teh et al. 2004)
I/II	Combination with IMRT Versus IMRT Alone for the Treatment of Newly-Diagnosed Intermediate-Risk PCa	Forty-four men with intermediate-risk PCa were randomly assigned to receive either OAMCGT plus IMRT or IMRT only. There was a 60% relative reduction in biopsy positivity. None of the patients developed hormone-refractory or metastatic disease and none have died from PCa. Now entered phase III clinical trials	I.T	CD/HSV/TK	(Freytag et al. 2014)
I/II	HSV-TK + Valacyclovir in combination with Brachytherapy for recurrent PCa without metastatic disease	Ongoing trial- Awaiting results	I.T	HSV/TK	
III	A Randomized Controlled Trial of ProstAtak? as Adjuvant to Up-Font Radiation Therapy for Localized PCa	Ongoing trial- Awaiting results	I.T	TK	

1.7 Research rationale

Localised PCa it currently treated in the UK through watchful waiting or active surveillance in order to avoid 'overtreatment', which can be incurred through invasive therapies, including radical prostatectomy and radiotherapy. However, as PCa progresses, treatment with ADT, including medical castration and AR antagonists, results in reactivation of the AR signalling pathway, leading to a hormone resistant cancer phenotype. Treatment of this castration resistant phenotype with chemotherapeutics is a palliative measure, eventually becoming ineffective, resulting in an urgent need for novel therapeutics that target CRPC. Many studies have demonstrated the efficacy of GDEPT and have moved on to restrict expression of this therapy through prostate TSPs, providing a safer treatment strategy for PCa. Whilst targeting GDEPT specifically to PCa cells improved efficacy, traditional prostate specific promoters were inherently weak, resulting in poor transduction. Steps were therefore taken to improve transduction through the use of TSTAs (Zhang, Adams, et al. 2002), and fusion of upstream regulatory elements, forming chimeric promoters (Latham et al. 2000, Schuur et al. 1996).

Whilst these later promoter systems have demonstrated some improved efficacy, they are relatively ineffective in androgen-independent cancer, due to the requirement for AR binding to AREs in their regulatory regions. *TMPRSS2* is an androgen regulated gene; its regulatory elements are commonly fused to *ERG* in 50% PCa cases (Tomlins et al. 2005), forming the TMPRSS2:ERG fusion, the most common fusion gene in human malignancy. The role of TMPRSS2:ERG in PCa progression has not yet been fully elucidated and contradictory reports suggest that it may be involved in malignant cell progression (Li et al. 2011, FitzGerald et al. 2008). Despite this, it is clear is that ERG expression through TMPRSS2:ERG fusion remains high in CRPC (Cai et al. 2009), suggesting that the regulatory elements of TMPRSS2 can still function throughout castration resistance. Utilising these regulatory elements to restrict GDEPT to PCa cells may offer a novel therapy that is functional in both localised and castration resistant PCas.

We therefore hypothesised that the regulatory elements of TMPRSS2 could offer a novel method with which to target PCa. We proposed to restrict the expression of

the *CD/UPRT* suicide gene through *TMPRSS2* regulatory elements, whilst additionally increasing transgene expression using a TSTA system. We therefore cloned the TMPRSS2-VISA-CD/UPRT expression cassette, whereby expression of the CD/UPRT suicide gene was under the control of the TMPRSS2 promoter region upstream of exon1, with enhanced transcriptional activation due to the VP16-GAL4-WPRE integrated systemic amplifier (TMPRSS2-VISA-CD/UPRT). We proposed to replace the adenoviral E1 and E3 regions with the 5.7 kb TMPRSS2-VISA-CD/UPRT expression cassette, producing a replication defective adenovirus that targets PCa cells specifically, in order to improve safety and transduction. To improve upon this system further, we aimed to clone a novel chimeric TMPRSS2 regulatory element, consisting of two regulatory elements upstream of Exon1 and Exon2 and compare its promoter activity to that of the more traditionally used PSA promoter. We further hypothesised that fusion of two regulatory elements of TMPRSS2 would drive higher levels of prostate specific expression than any previously validated TSP.

1.7.1 Previous work

Three putative *TMPRSS2* promoters were identified from a combination of a review of the literature (Wang et al. 2007, Lin et al. 1999) (Figure 24) and use of the online software tool Genomatix promoter prediction (http://www.genomatix.de). The three promoters were inserted into the VISA vector (a kind gift from Professor Hung, MD Anderson, TX, USA), replacing the CCKAR promoter, and named L-VISA, W-VISA, and G-VISA (Table 11) (Kevin Sharp, our team, unpublished data). Tissue specific expression and promoter activity was analysed in these vectors by employing the luciferase gene as the gene of interest in 22RV1, HEK293, PC3 and LNCaP cells.

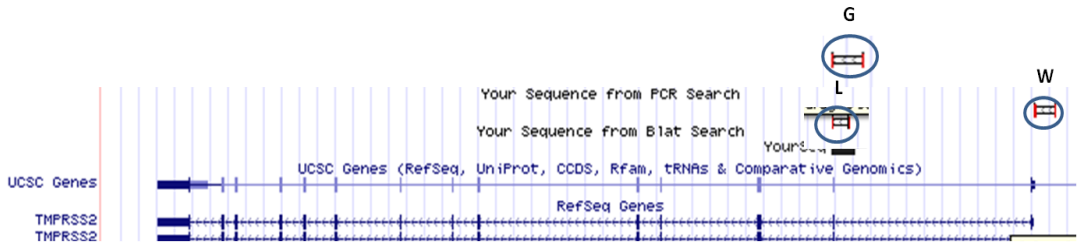


Figure 24 Genomic position of each of the *TMPRSS2* promoters used.

L-VISA (L) sequence position circled upstream of exon 2 (horizontal line) G-VISA (G) sequence position circled upstream of exon 2 (horizontal line) W-VISA (W) sequence position circled upstream of Exon1 (horizontal line).

Table 11 Three *TMPRSS2* promoters explored in the VISA-amplification vector and the respective location on chromosome 21.

Construct name	Source	Description	Position on Chr:21	Length (bp)
G-VISA	Genomatix software	Promoter region upstream from Exon2	41791831-41792406	576
L-VISA	Lin et al (Lin et al. 1999)	Promoter region upstream from Exon2	41791861-41793048	1,188
W-VISA	Wang et al (Wang et al. 2007)	Promoter region upstream from Exon1	41801925-41802935	1,011

The W-VISA promoter demonstrated the strongest activity in all cell lines tested, including the AR-positive cell line LNCaP. However, this promoter specificity was compromised due to non-specific luciferase expression detected in HEK293 and PC3 cells. L-VISA appeared to be the most promising candidate as it showed tissue specificity in the AR-positive PCa cell line 22RV1, along with significantly stronger expression of luciferase than G-VISA in 22RV1 cells; however this was not the case in any other cell line (Table 12).

Table 12 Luciferase activity of the three *TMPRSS2* promoter regions inserted in the VISA-vector and transfected into PCa and non-PCa cell lines.

Vector	Cell line	Mean	Standard Deviation	t-stat (versus HEK293)	p-value (versus HEK293)
G-VISA	22RV1	248	55	10.8	0.0001
	LNCaP	5	3	0.4	0.3457
	PC-3	1	1		
	HEK 293	5	2		
L-VISA	22RV1	362	84	10.4	0.0001
	LNCaP	7	4	0.9	0.1855
	PC-3	1	1		
	HEK 293	5	2		
W-VISA	22RV1	771	223	4.8	0.0011
	LNCaP	257	148	0.5	0.3061
	PC-3	122	68		
	HEK 293	295	95		

Mean values and standard deviation measurements refer to firefly luciferase/ renilla luciferase. Values are representative of two independent experiments, each consisting of triplicate wells. t-stat and p-value assess whether there is a statistically significant difference between the expression levels from 22RV1 and LNCaP and the most active negative control, HEK 293, using each of the different VISA constructs.

L-VISA was taken forward for further studies because it showed the most promising specificity, while still driving high levels of luciferase expression. The

poor luciferase expression in LNCaP cells was assumed to be due to difficulties in transfection of this cell line. The luciferase gene was replaced with the *CD/UPRT* suicide gene and cell specific killing was demonstrated upon the addition of 5-FC pro-drug in 22RV1 cells alone (Ahmet Imrali, our team, unpublished observations) (Figure 25).

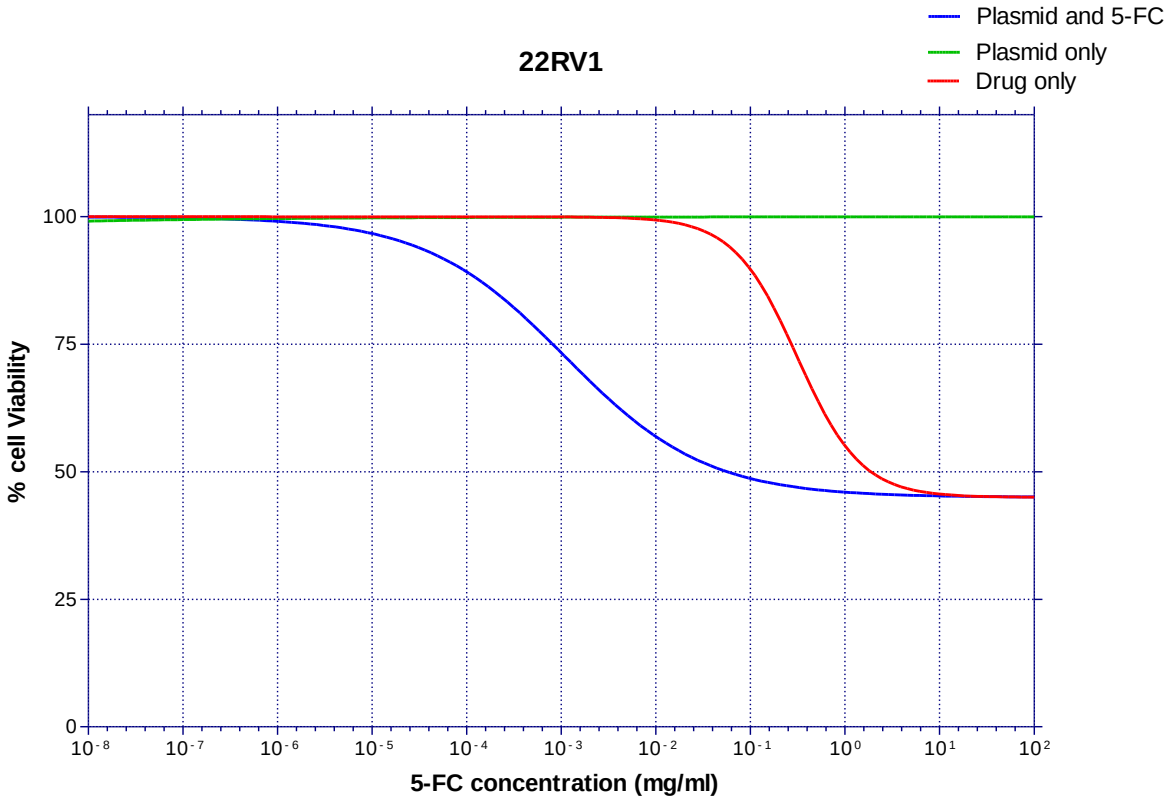


Figure 25 Decrease in cell viability after addition of pro-drug in combination with the L-VISA plasmid

The L-VISA plasmid was transfected into 22RV1 cells and treated in combination with three different doses of drug at 0.1, 0.01 and 0.001 mg/ml over six replicates. Cell viability was measured after 72 hours by MTS assay. Cell viability decreased with increasing concentration of 5-FC. Data generated by Ahmet Imrali, our team, unpublished observations.

Ad5-TV-CU was constructed (Ahmet Imrali, our team, unpublished observations), but showed no expression of CD/UPRT protein after infection in 22RV1 or LNCaP cells, as well as no dose dependent shift in EC_{50} values when the virus was combined with 5-FC.

In summary these results demonstrate that the TMPRSS2 promoter can be utilised to drive high level prostate specific expression of a luciferase transgene. Replacement of the luciferase transgene with the *CD/UPRT* gene and addition of

increasing doses of 5-FC drove enhanced prostate cell specific killing in a 5-FC dose dependent manner. This evidence suggested that this system could be utilised as a novel enzyme prodrug therapy for the treatment of PCa.

1.8 Aims of this thesis

This research project was conducted with the aim of utilising the AR regulated *TMPRSS2* promoter to drive tissue specific suicide gene therapy in PCa cells. Specific systems to enhance the promoter activity and the ability to deliver the gene into cells were developed to increase transduction and efficacy of the therapy. Based on previous unpublished findings generated in our team the following steps to produce the optimal therapy were investigated.

1. Exploring the potential for amplification of the optimal *TMPRSS2* promoter construct, by insertion in the VISA amplification system and using a replication-deficient adenovirus for efficient transduction in all available PCa cell lines, and transformed prostate cells.
2. Comparing the *TMPRSS2* promoter to other prostate-specific promoters, including the 'classical' *PSA* promoter constructed in the same system.
3. Investigating the cell line specificity and 5-FC dose dependent activity of this gene directed enzyme prodrug therapy using a replication-deficient virus construct *in vitro,* including castration-resistant LNCaP-CDXR3 and LNCaP-104R1 cells.
4. Investigating the efficacy of this replication-deficient virus *in vivo* in combination with 5-FC.
5. Comparing a range of *TMPRSS2* promoter regions and producing chimeric promoters to increase specific promoter activity.

Therefore, in this thesis I produced and characterised the replication deficient Ad5-TMPRSS2-VISA-CD/UPRT (Ad5-TV-CU) virus, whereby transgene expression was placed under the control of *TMPRSS2* regulatory elements to drive prostate specific expression of the *CD/UPRT* suicide gene. This PCa specific enzyme prodrug therapy was evaluated *in vitro* and *in vivo* for efficacy and prostate specificity. I also sought to clone a new chimeric *TMPRSS2* promoter, with superior promoter activity and prostate specificity compared to the previously validated *TMPRSS2* and *PSA* promoters.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and cell culture.

2.1.1 Cell Culture.

The PCa cell lines, 22RV1, LNCAP, VCaP, DU145 and PC3 were obtained from American type culture collection (ATCC). Normal human prostate epithelial cell lines (PrEC) were purchased from LONZA. Two SV40-immortalised prostate epithelial cell lines PNT1a and PNT2 were obtained from Norman Maitland and Colin Cooper, respectively. The LNCaP sublines LNCaP-104-S (androgen-sensitive) and the androgen ablation-resistant and bicalutamide-resistant cells LNCaP-104-R1 and LNCaP-CDXR3 respectively, were a generous gift from Dr John M. Kokontis (University of Chicago, Chicago, USA). Molecular status of these cell lines, including p53, *TMPRSS2*:*ERG* fusion, and cell type are described in Table 13.

The transformed human embryonic kidney (HEK293) cell line was used for primary viral expansions, as well as a negative control for transfection experiments, as they do not express the androgen receptor (AR). These cells have been transformed with the first 4344 nucleotides of Ad5, and therefore express the E1 gene, providing the basis for viral replication of mutants not expressing E1A. JH293 cells are a sub-clone of HEK293s and were used for tissue culture infected dose ($TCID_{50}$) assays. Both HEK293 and JH293 were obtained from Cancer Research UK Cell Services. All experiments were carried out using cells between 3 and 30 passages. All PCa cells, as well as HEK293 and JH293 cells, were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (PA laboratories UK), with the exception of PC3, PrEC, LNCaP-CDXR3 and LNCaP-104-R1. PC3 cells were grown in Roswell Park Memorial Institute (RPMI) supplemented with 10% FBS. The LNCaP cell lines LNCaP-104-R1 and LNCaP-CDXR3 were cultured in DMEM supplemented with 10% charcoal-stripped FBS (Sigma Aldrich). All three LNCaP cell lines were supplemented with 0.2nM DHT. Medium for LNCaP-104-S, LNCaP-104-R1 and LNCaP-CDXR3 cells was changed every 2-3 days if cells were not passaged. The normal PrEC cells were grown in specialised media (PrEBM) according to the manufacturer's instructions (Lonza). All cells were grown at 37°C in a humidified atmosphere with 5% CO_2. All cell lines were short tandem repeat (STR) profiled to

confirm the cell line and vials of STR profiled cells frozen down and stored in liquid nitrogen to form long term stocks and avoid cross-contamination.

22RV1 cells obtained from ATCC have previously been shown to test positive for the presence of replication-competent xenotropic murine leukemia virus-related virus (XMRV) (Knouf et al. 2009). XMRV is associated with prostate carcinogenesis most frequently in men with a defect in the antiviral defense protein RNase L. This discovery could potentially impact the interpretation of experimental results.

2.1.2 Cell storage.

Cells were stored in liquid nitrogen in 10% dimethyl sulfoxide (DMSO), 20% FCS and 70% respective culture medium. PrEC cells were stored in 10% DMSO, 10% FCS and 80% clonetics PrEBM media (Lonza). Cells were revived by thawing in a 37°C water bath and immediately adding in a drop wise manner to 10 ml full (10% FCS, 1% penicillin/streptomycin) pre-warmed DMEM (22RV1, LNCaP, DU145, VCaP, HEK293, LNCaP-104-S), full RPMI (PC3), PrEBM (PrEC cells), or DMEM containing 10% charcoal stripped media (LNCaP-CDXR3 and LNCaP-104-R1). The cells were pelleted by centrifuging at 1200 rpm for 5 min and then resuspended in 2 ml of culture media. All cells were then added to a T75 flask containing 10 ml of pre-warmed culture media and incubated under standard conditions (37ºC humidified atmosphere with 5% CO_2).

2.1.3 Cell maintenance.

Cells were sub-cultured every 3-5 days, or when they reached 80-90% confluency, by washing with 10 ml phosphate buffered saline (PBS) and addition of 2 ml 1x trypsin EDTA to detach cells. Trypsin was deactivated by the addition of 5 ml of culture media. The cells were finally split 1 in 5 by taking $1/5^{th}$ of the cell suspension and adding to a new flask with fresh culture media (22RV1, DU145, HEK293, PC3, PNT2, PNT1A LNCaP). VCaP cells were cultured every 7 days using the same protocol but splitting the cells only 1:3. PrEC cells were subcultured every 7 days with media changes every 3 days. Cells were washed with 10 ml hepes buffered saline solution and then 3 ml 1x trypsin EDTA was added to detach the cells. Trypsin was deactivated by adding 6 ml trypsin neutralising solution (TNS) and the cell pellet spun down at 1200 rpm for 5 min.

The cell pellet was resuspended in 6 ml of PrEBM media, and the cells split 1:3 into flasks containing pre-warmed PrEBM.

2.1.4 Cell counting.

Cells were left to grow to 80% confluency and trypsinised. The trypsin was neutralised by addition of 5 ml 10% FCS DMEM/RPMI and spun at 1200 rpm for 5 min. The cell pellet, $6x10^6$ cells, was resuspended in 20 ml of respective culture media and 10 µl of cell suspension added to 10 µl of trypan blue (Sigma Aldrich, USA). 10 µl of the solution was added to a cell counting slide, counted using the biorad cell counter system and seeded at the appropriate density for the experiment using the following equation.

$$\frac{Total\ number\ of\ cells}{Number\ of\ cells\ needed} = dilution\ factor$$

$$\frac{Total\ cell\ suspension\ vol}{dilution\ factor} = mls\ cells$$

Table 13 Cells status and origin

	22RV1	VCaP	DU145	PC3	LNCaP	PrEC	PNT2	PNT1A
Origin	Primary tumour from CRW33Rv xenograft serially passaged in mice	Metastatic tumour in the lumbar vertebral body xenografted on SCID mice	Carcinoma brain metastasis	Grade IV adenocarcinoma bone metastasis	Carcinoma supraclavicular lymph node metastasis	Primary prostate epithelial cells	Normal prostate epithelium immortalized with SV40.	Normal prostate epithelium immortalized with SV40.
Androgen dependency	A.I Sensitive	A.I Sensitive	A.I	A.I	A.D Sensitive	A.D	N/A	N/A
TMPRSS2:*ERG*	-	+	-	-	-	-	-	-
AR status	Three mutated types full length AR Ex^{3dup} and constitutively active truncated $AR^{1/2/2b}$ and $AR^{1/2/3/2b}$	Wild type amplified gene locus and $AR^{1/2/2b}$	-	-	Full length mutated T877A converts antagonist flutamide into agonist	Wild type	Wild type	Wild type
p53 status	Mutated (Q331R)	Mutated (A248W)	Mutated (P223L and V274F)	Stop codon at 169	Wild type	Wild type	Wild type	Wild type
PTEN	+	+	+	-	-	+	+	+
AKT	+	+	+	+	+	+	Unknown	Unknown
BCL-2	++	+	-	+	+	Unknown	-	Unknown
Established	1999	2001	1978	1979	1980	N/A	1995	1991
Reference	Sramkoski, Pretlow et al. 1999	Korenchuk S et al 2001	Stone, Mickey et al. 1978	Kaighn, Narayan et al. 1979	Horoszewicz, Leong et al. 1980	(Ltd 2014)	(Berthon et al. 1995)	(Cussenot et al. 1991)

+=positive - = negative A.I= androgen-independent, A.D= androgen-dependent

2.2 Cloning.

2.2.1 PCR amplification of DNA

20 ng of DNA was added to 1 µl 10 mM dNTPs, 5 µl 10x PCR buffer, 1 µl forward primer (10µM), 1 µl reverse primer (10µM) and 2 units taq polymerase. The final volume was made up to 50 µl with water and underwent 35 cycles of PCR.

94 °C 5 min

94 °C 30 sec
60 °C 45 sec
72 °C 2 min
} X34

72 °C 2 min

2.2.2 Restriction digest

5 µl of DNA was added together with 10 units (1 µl) of each of the required restriction enzymes (New England biolabs, (NEB), Table 16). 3 µl of 1x BSA was added to each reaction as well as 3 µl of the specific 10x buffer for the enzyme, 1, 2, 3 or 4 (NEB). The final volume was made up to 30 µl with water and left to digest at 37°C for 2 h. If the product then required blunting 4 µl 10 mM dNTPs was added with 1 µl blunt enzyme mix (100 mM KCl, 10 mM Tris-HCl(pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100 and 50% Glycerol) and 10mM dithiothreitol (DTT) and incubated at room temperature for 15 min and subsequently heated to 70°C to inactivate the enzymes. Digested vectors were additionally treated with Antarctic phosphatase by addition of 3 µl Antarctic phosphatase buffer and 1 µl Antarctic phosphatase (NEB) to remove 5' phosphatase groups, which consequently prevented self-ligation of the vectors. Digested products were then run on a gel for gel extraction or DNA product size analysis.

2.2.3 Phenol:chloroform clean-up of DNA

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA solution, mixed well and centrifuged at 10,000 rpm for 3 min. The aqueous layer was transferred to a new tube and an equal volume of chloroform added,

mixed and centrifuged at 10,000 rpm for 3 min. The aqueous layer was again removed and 2.5 volumes of 100% ethanol and 1/10 volumes of 3 M NaAc added to the DNA and kept overnight (O/N) at -20°C. The solution was centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant decanted. 1 ml of 70% ethanol was added and incubated at room temperature (RT) for 5 min. The solution was centrifuged at 14,000 rpm at 4°C for 20 min, the pellet was dried and then resuspended in 30 µl of dH_2O.

2.2.4 Gel extraction protocol

The gel was placed on a UV light box in the dark room and the DNA cut from the gel using a sharp scalpel. DNA was extracted using the QIAQUICK gel extraction kit (Qiagen, Germany). Briefly, the gel piece was weighed and three times the volume of buffer QG added to one volume of the gel. The gel was then warmed at 50°C until it dissolved into the buffer. 1x gel volume of isopropanol was added and mixed and the solution added to a QIAquick column. The DNA was bound to the column by centrifugation at maximum speed for 1 min. The column was washed with 750 µl of buffer PE and centrifuged for 1 min. The DNA was then eluted into a new microcentrifuge tube by addition of 50 µl of dH_2O.

2.2.5 Cracking gel protocol

1 ml bacterial growth was spun down at 14,000 rpm. Cell pellets were resuspended in dH_2O and a standard phenol/chloroform extraction protocol performed to obtain plasmid DNA. 5 µl DNA was loaded onto a gel and potential recombinants identified due to size differences. Potential recombinants were miniprepped (section 2.4.6) for further analysis.

2.2.6 Miniprep

Wizard® *Plus* SV Minipreps DNA Purification System, USA was used to miniprep bacterial DNA. 1-5 ml of bacteria were spun down and resuspended in 250 µl of cell resuspension solution and 250 µl of cell lysis solution added and mixed. 10 µl of alkaline protease was then added and incubated for 5 min at RT. 350 µl cell neutralising solution was added, inverted 4 times, and centrifuged for 10 min at RT. The cleared lysate was decanted into the spin column and centrifuged at top speed for 1 min at RT. 750 µl column wash solution was added and the tube centrifuged for 1 min. A further 250 µl column wash solution was added and the

tube centrifuged for 2 min. The column was transferred to a new eppendorf and 50 µl dH_2O added, the tube was spun for 1 min at RT to elute the bound DNA.

2.2.7 Blue/white colony screening

1 µl of PCR product, 2 µl of T4 DNA ligase reaction buffer, 2 µl pCR2.1 vector and 1 µl express link T4 DNA ligase was made up to a total volume of 10 µl with sterile water. The ligation mixture was left at RT for 30 min and then transformed into 25 µl TOP10 chemically competent cells (Invitrogen) and placed on ice for 5 min. Meanwhile, agar plates containing 100mg/ml ampicillin were spread with 40 µl of x-gal (40 mg/ml) and left to dry. After 5 min the transformed cells were immediately spread on the agar plates (ampicillin resistance gene is immediately expressed), the plates were inverted and incubated O/N at 37˚C. The following day white colonies (those containing the insert due to interruption of the lacZ gene) were picked and grown in lysogeny broth (LB) containing ampicillin, ready for miniprep and restriction digestion analysis.

2.3 The VISA plasmids.

The PSA promoter was cut directly from the pDRIVE-PSA-hPSA plasmid (Invivogen) using Nco1 and Spe1 restriction enzymes and the PSA promoter/enhancer were PCR amplified from pDRIVE-PSA-hPSA using primers with SmaI and PmeI restriction sites (Table 14). The DNA was run on an agarose gel, extracted (section 2.4.4) and blunted by adding 4 µl 10 mM dNTPs, 1 µl blunt enzyme mix and 10mM DTT, incubated at RT for 15 min and ligated into E-VISA (empty VISA expression cassette) using the 3:1 cloning ratio (see below), producing P-VISA and PE-VISA.

$$Weight\ of\ insert = Weight\ of\ vector\ \frac{Insert\ size}{Vector\ size} x\ \frac{3}{1}$$

Plasmids were then sequenced using the primers listed below (Table 14)

Table 14 PSA VISA plasmids sequencing primers

PE-VISA sequencing	P-VISA sequencing
PSA1 TGAATGGCTGGGATGTGTC	F2 VISA TCAGTAGAAATAGCTGTTCCAGTC
PSA2 CTGGGTCCCCTCCTATCTCT	PSA2 CTGGGTCCCCTCCTATCTCT
PSA3 TGATCTTGGATTGAAAACAGACC	
PSA4 TTCTAGGTCCCGATCGACTG	
PSA5 GCACGTGAGGCTTTGTATGA	

2.3.1 The PGL3 plasmids.

The N-PGL3 plasmid was previously made by ligating the *NKAIN2* promoter into the empty PGL3 plasmid (our lab, Lara Boyd). S-PGL3 is a commercial plasmid containing the SV40 promoter (Promega, USA). The remaining PGL3 plasmids were made by PCR amplifying the various *TMPRSS2* promoter elements from a combination of the L-VISA vector, the W-VISA vector and genomic DNA, using primers with appropriate flanking restriction sites (Table 15). The DNA was run on an agarose gel, extracted (section 2.2.4) and ligated into the pCR2.1 vector (Invitrogen). Colonies containing the inserts were miniprepped and sent for sequencing. The PCR products were digested from the pCR2.1 vectors using the appropriate restriction enzymes and ligated into complementary sites in the E-

PGL3 plasmid (Table 16) using the rapid DNA ligation kit (Roche, Germany). Briefly, 5 µl of DNA ligation buffer, 1 µl DNA ligase and 1 µl of both the vector and insert were mixed in an eppendorf and left at RT for 5 min. Subsequently, the entire 10 µl of ligation mixture was added to 50 µl of TOP10 chemically competent cells and spread on agar plates containing 100 mg/ml ampicillin. Plates were incubated overnight. The following day colonies were picked and grown in LB containing ampicillin ready for miniprep and restriction digestion analysis (section 2.2.2). Potential recombinants were sent for sequencing to confirm inserts using the primers listed in Table 17.

Table 15 PCR primers for amplifying TMPRSS2 promoter DNA and PSA promoter and enhancer DNA.

Construct	F primer	R primer
LLW-PGL3	AAGCTTAGGACAACAAGCAAAATGGC	AAGCTTCCCTCCGCCTCCTGCTTAG
PPE-PGL3	GGTTTTTTGTTGGTACCCCTGCAGGCCTCT	ATGAGAACCCCTCGAGGTGACACAGCTC
P-PGL3	CTCAGGAGGTACCCTAGTACATTGTTT	ATGAGAACCCCTCGAGGTGACACAGCTC
PEW-PGL3	GGTTTTTTGTTGGTACCCCTGCAGGCCTCT	CAAACAATGGGTACCTCGGGATCCT
PEL-PGL3	GGTTTTTTGTTGGTACCCCTGCAGGCCTCT	CAAACAATGGGTACCTCGGGATCCT
L-PGL3	GGTACCAGGACAACAAGCAAAATGGC	AAGCTTCAGAAGGGACAAGGGAACAA
PE-VISA	GGGTTTTTTGTTCCCGGGCCTGCAGGCCTCT	GAGCTGTGTCACGTTTAAACGTTCTCATCAT
LW-PGL3	TGAACGGTACCTGCCGTGTGAGGCAGATAA	GTTGGAAGCTTCAGAAGGGACAAGGGAACA
WE-PGL3	CATTGCAATAAGAACTTC	GCCTTGTGACACTTCACCC
PE-VISA	GGGTTTTTTGTTCCCGGGCCTGCAGGCCTCT	GAGCTGTGTCACGTTTAAACGTTCTCATCAT
W-PGL3	GGTACCTGCCGTGTGAGGCAGATAA	AAGCTTCCCTCCGCCTCCTGCTTAG

Table 16 Restriction enzymes used for cloning and the vectors, insert and new plasmid information

Plasmid to be generated	Original plasmid digestion	PCR product digestion	Vector digestion
P-VISA	pDRIVE PSApEnh NcoI/SpeI	N/A	N-VISA EcorV/SpeI
PShuttle-TMPRSS2-VISA-CD/UPRT	L-VISA SalI/NotI	N/A	pShuttle SalI/NotI
PShuttle CMV-GFP	pE*GFP*–C2 AseI and MluI	N/A	PShuttle EcorV
pAdEasy-*TMPRSS2*-VISA-*CD/UPRT*	PShuttle-TMPRSS2-VISA-CD/UPRT PmeI	N/A	pAdEasy- No digestion
PE-VISA	N/A	pDRIVE PSApEnh PCR product digested SmaI/PmeI	N-VISA EcorV/SpeI
L-PGL3	N/A	L-VISA PCR product digested KpnI/HindIII	PGL3 KpnI/HindIII
E-VISA	N-VISA Ecorv/SpeI	N/A	N/A
PW-PGL3	N/A	PSA enhancer Kpn1	W-PGL3 Kpn1
PL-PGL3	N/A	PSA enhancer Kpn1	L-PGL3 Kpn1
LLW-PGL3	N/A	LW HindIII	L-PGL3 HindIII
PEP-PGL3	N/A	PSA enhancer/promoter Kpn1/XhoI	PGL3-B KpnI/XhoI

PP-PGL3	N/A	PSA promoter Kpn1/XhoI	PGL3-B KpnI/XhoI
W-PGL3	N/A	W-VISA PCR product digested Kpn1/HindIII	PGL3 KpnI/HindIII
LW-PGL3	L-PGL3 Kpn1/Hind III and blunted	N/A	W-PGL3 kpn1 and blunted
EW-PGL3	W enhancer Kpn1/BglII and blunted	N/A	W-PGL3 Kpn1 and blunted
EL-PGL3	W enhancer Kpn1/BglII and blunted	N/A	L-PGL3 Kpn1 and blunted

Table 17 PGL3 plasmid sequencing primers

PGL3 plasmid sequencing			
All PGL3 plasmids	RV primer 3 CTAGCAAAATAGGCTGTCCC	LLW-PGL3	R2 CACTCTCCCACAACCCTC
	PGL2 TGGAAGACGCCAAAAACATAAAG		R3 CGCTGCACTTACAATTGC
L-PGL3	VISA TTTGGGACCCACTTGTG		LW-PGL3 GTCCGGGAACAGCTCTCTT
	VISA AAGACCTGGGCCAGTTC	PW-PGL3	PSA1 TGAATGGCTGGGATGTGTC
	VISA TCAGAGCCAACCATTTTG		PSA2 CTGGGTCCCCTCCTATCTCT
LW-PGL3	LW-PGL33 CACAAGTGGGTCCCAAA		PSA3 TGATCTTGGATTGAAAACAGACC
	LW-PGL3 GTCCGGGAACAGCTCTCTT		PSA4 TTCTAGGTCCCGATCGACTG
	LW-PGL32 GAACTGGCCCAGGTCTT	PL-PGL3	PSA1 TGAATGGCTGGGATGTGTC
EW-PGL3	WPE-PGL3 GGGTGACAGAAAGGAATGGG		PSA2 CTGGGTCCCCTCCTATCTCT
	WPE- PGL3 CCCATTCCTTTCTGTCACCC		PSA3 TGATCTTGGATTGAAAACAGACC
EL-PGL3	EL-PGL3 GGGGTTTGAGGCCTCTGG		PSA4 TTCTAGGTCCCGATCGACTG
	EL-PGL3 CCAGAGGCCTCAAACCCC		F3 TTTGGGACCCACTTGTG
LLW-PGL3	LLW-PGL3 CACTCTCCCACAACCCTC	PPE-PGL3	PSA1 TGAATGGCTGGGATGTGTC
	LLW-PGL3 CGCTGCACTTACAATTGC		PSA2 CTGGGTCCCCTCCTATCTCT
	LW-PGL3 GTCCGGGAACAGCTCTCTT		PSA3 TGATCTTGGATTGAAAACAGACC
PW-PGL3	PSA1 TGAATGGCTGGGATGTGTC		PSA4 TTCTAGGTCCCGATCGACTG
	PSA2 CTGGGTCCCCTCCTATCTCT		PSA5 GCACGTGAGGCTTTGTATGA
	PSA3 TGATCTTGGATTGAAAACAGACC		
	PSA4 TTCTAGGTCCCGATCGACTG		

2.4 Protein expression analysis.

2.4.1 Plasmid transfection

Cells were seeded at $1.5x10^5$ (LNCaP, PC3, DU145) and $3x10^5$ (22RV1, VCaP, PNT1A, PNT2) in 2 ml respective culture media (10% FCS, 1% pen/strep) in 6 well plates. The following day the media was replaced with 1 ml 2% respective culture media (2%FCS, 0% pen/ strep). 2 µg of plasmid DNA per well was mixed with 125 µl 0% respective culture media (0% FCS, 0% pen/ strep). 5 µl lipofectamine (Invitrogen) was mixed separately with 125 µl 0% respective culture media per well. The lipofectamine and DNA solutions were combined and incubated for 30 min at RT, in order for the DNA/lipid complexes to form. After incubation the full 250 µl was added to the 6 well plates containing cells. 4 h later 1 ml of the respective full media was added to each well. Cell lysates were extracted at 24, 48 and 72 h post transfection for protein and/or RNA analysis.

2.4.2 Preparation of protein lysates.

Cells were lysed in 1x LYSIS buffer containing 1x protease inhibitor cocktail (Roche, Diagnostics, Mannhein, Germany), 1% triton-x-100 and PBS. 200 µl was added to $3x10^5$ cells. Lysates were centrifuged at 14,000g for 15 min at 4°C. The supernatant was transferred to a new Eppendorf and stored at -80°C. Protein concentrations were determined by Bradford assay (BioRad, Philadelphia, PA, USA) using bovine serum albumin (BSA) (NEB) stock at 1 µg/µl to make a standard curve from 0 to 1 µg. Proteins were diluted 1:10 and 10 µl added to 190 µl of 1x BioRad reagent (5x stock diluted with dH_2O) in duplicate. Absorbance was measured by the Opys 96-well microplate reader (DYNEX) spectrophotometer at 595 nm and the protein concentrations calculated based on the standard curve generated by the absorbance of BSA standards.

2.4.3 Western Blotting.

Total protein (20 µg) from cell lysates was mixed with 7.5 µl 1X loading buffer (NuPAGE LDS Sample Buffer; Novex) and 3 µl 1X reducing buffer (NuPAGE Sample Reducing Agent; Invitrogen, UK), and incubated at 95ºC for 5 min to denature the proteins. 30 µl was loaded onto 10% sodium dodecyl sulphate reducing polyacrylamide gels (SDS-PAGE), together with 10 µl of spectra

multicolour broad range protein ladder (Fermentas, UK) to determine protein size. The proteins were separated by electrophoresis at 120 V in running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS, pH 8.3). Proteins were transferred to polyvinylidene fluoride membranes (PVDF) with a 0.45 µm pore size (Millipore) by a wet transfer technique, at 200 mA for 100 min in transfer buffer (10% 25 mM Tris-250 mM glycine, 20%, Methanol, pH 8.5). Membranes were blocked with 5% (w/v) dry milk (Marvel, Dublin, Ireland) in Phosphate-buffered saline, Tween-20 buffer (PBS-T; 20 mM Tris-HCl pH 7.4, 500 mM NaCl) with 0.05% Tween-20. After blocking, membranes were washed 3x 10 min in 0.05% PBS-T. The membranes were incubated overnight at 4°C with primary antibodies (Table 18) in 5% BSA (Sigma) in PBS-T, washed and incubated with secondary antibodies (Table 19), conjugated to horseradish peroxidase (DakoCytomation) in 5% (w/v) dry milk in PBS-T for 1 h at RT. The membranes were washed 3x 10 min with PBS-T after each antibody incubation. Proteins were visualised using Immobilon Western Chemiluminescent HRP Substrate (Millipore), by mixing equal volumes of luminol reagent and peroxide solution and incubating the membrane in the solution for 5 min at RT. Finally, the blot was exposed to autoradiography films (Fujifilm Düsseldorf, Germany) for 30 s-5 min and developed in a Curix 60 Developer (Agfa, Middlesex, UK).

Table 18 List of primary antibodies used for western blotting

Protein	Species and clone	Catalogue number	Company	Dilution
Cytosine deaminase	Polyclonal Sheep	ab35251	Abcam	1:500
AR	Polyclonal Rabbit-N20	sc-816	Santa Cruz	1:300
β-actin	Monoclonal Mouse	A5441	Sigma Aldrich	1:10,000

Table 19 List of secondary antibodies used to detect primary antibodies in Western blotting

Species and clone	Catalogue number	Company
Rabbit anti-sheep	p0163	Dako
Goat anti-mouse	32430	Thermo scientific
Goat anti-rabbit	32460	Thermo scientific

2.4.4 Transgene expression in response to mibolerone/ Bicalutamide

One day before seeding, cell culture medium was changed to 10% charcoal-stripped FBS-containing media to remove residual hormones. 22RV1 and LNCaP cells were seeded in 96-well plates at $2x10^4$ and $1x10^4$ respectively, in 10% charcoal-stripped FBS, DMEM. The following day the media was removed and replaced with 90 µl charcoal-stripped FBS DMEM. Cells were infected at fixed doses of Ad5-TV-CU at 1000, 2000, 3000 ppc. 2 h later a fixed concentration of either mibolerone or Bicalutamide at 1nM and 20µM respectively was added to the cell. 48 hours after the combined treatment cells were lysed for western blot analysis of CD/UPRT protein expression.

2.5 Cell viability assays.

2.5.1 Determining Ad5-TV-CU EC_{50} values

$1x10^4$ cells/well (DU145, LNCaP, PC3 and PNT2) or $2x10^4$ cells/well (22Rv1, VCaP and PNT1A) were seeded in 96-well plates in a volume of 200 µl/well of their respective culture media (10% FCS 1% Pen/Strep). PrEC ($5x10^4$ cells/well) were seeded in 96-well plates in a volume of 200 µl/well of PREBM media. The next day the medium was replaced with 90 µl/well (2%FCS 1%Pen/Strep). An initial viral dose of $1x10^5$ ppc was made and serially diluted 5-fold in serum-free media. 10 µl of the viral dilutions were added to the wells, therefore the viral dilution was initially made at 10x concentration to account for the further 1/10 dilution in the wells of the plate. Viability was assessed 3 days post-treatment using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) viability assay, carried out according to the manufacturer's protocol (Promega, WI, USA). Briefly, media was removed and 100 µl MTS reagent added. Phenazine methosulfate (PMS) is added to MTS, and in the presence of live cells (the mitochondrial NAD(P)H-dependent cellular oxidoreductase enzyme) the tetrazolium salt is converted to a formazan product that absorbs light at 490-500 nm. The absorption is proportional to the number of live cells, as the production of formazan relies on the mitochondrial dehydrogenases present only in live cells. After addition of the MTS reagent, cells were incubated at 37ºC for 5 h (22Rv1), 2h (DU145, PC3, PNT1A, PNT2) or 9 h

(VCaP) and absorbance read using a microplate reader (Opsys, MR, Dynex Technologies) at a wavelength of 495 nm. Control wells containing only media were used to subtract background and untreated wells were used as positive controls. The percentage cell death for each treatment was calculated using the untreated control cells as 100% live cells. Sigmoidal dose-response curves were generated by non-linear regression analysis using GraphPad Prism Version 5.03 (GraphPad Software, San Diego, CA, USA) to determine the effective concentration (EC_{50} values) required to kill 50% of cells.

$$\%\ Cell\ death = 100 - \left\{\frac{Abs\ sample - Abs\ medium}{Abs\ untreated - Abs\ medium}\right\}$$

2.5.2 Determining effective concentration of 5-FC and 5-FU drugs

$1x10^4$ cells/well (DU145, LNCaP, PC3 and PNT2) or $2x10^4$ cells/well (22Rv1, VCaP and PNT1A) were seeded in 96-well plates in a volume of 90 µl/well of their respective media (10% FCS 1% Pen/Strep). Cells were treated with 5-fold serial dilutions of the non-toxic pro-drug 5-FC and the toxic metabolite 5-FU at a starting dose of 10 mg/ml in serum free media. The drugs were made up at 10-fold higher concentration to allow for the dilution when added to the plate, 10 µl of each dilution was added to 90 µl medium in the well in duplicate or triplicate. Medium alone and untreated cells served as controls. Cell viability was determined by MTS three days after treatment (section 2.5.1).

2.5.3 Combination of virus and 5-FC

Once the dose limiting toxicity of 5-FC was established, cells were seeded in 80 µl/well where both virus and drug were added in combination and 90 µl/well where only one treatment was given. Ad5-GFP was used as a negative control. Viruses were serially diluted 5-fold from 10^5 ppc and 5-FC added at selected fixed concentrations that were non-toxic to the cells, selected as a result of analysing EC_{50} values for titrations of drug alone. Cell viability was assessed by MTS assay 3 days post treatment as described in section 2.5.1. Results were corrected against background wells containing medium only and expressed as a percentage of cell death compared with untreated control cells for virus alone, or compared with drug treatment alone for combination of virus and 5-FC.

2.6 Viruses.

2.6.1 Adenoviruses.

The pAdEasy adenoviral backbone plasmid and the pShuttle plasmid (Dr Halldén, BCI) were used to clone Ad5-TV-CU (section 2.6.2). Ad5-GFP was non-replicating; with the E1 genes replaced by a CMV-GFP cassette, but still contained the E3 genes. Ad5-GFP and Ad5 wild type (wt) were obtained (Dr Halldén, BCI) and had previously been characterised by Dr Halldén's team, using specific PCR primer sets for identification and assays to determine particle and pfu section 2.7.1-2.7.2, Table 20).

Table 20 Viruses used and transgene expression

Virus	Characteristics	Transgene expression
Ad5-TV-CU	*TMPRSS2*-VISA-*CD/UPRT* replacing E1. Non-replicating.	*CD/UPRT*
Ad5-GFP	CMV-GFP replacing E1 genes. Non-replicating	GFP
Ad5wt	-	-

2.6.2 Constructing a prostate specific Ad5-TV-CU

The pAdEasy system (Stratagene, CA, USA) was used to generate Ad5-TV-CU. The TMPRSS2-VISA-CD/UPRT expression cassette was first cloned into the pShuttle vector (Stratagene, CA, USA) (section 2.6.2.1), linearised with PmeI and electroporated with pAdEasy into BJ5183 cells (section 2.6.3). Recombinant colonies were selected for by restriction digestion and plasmids purified. Recombinant pAdEasy was digested with Pac1 to expose its inverted terminal repeats (ITR) and transfected into HEK293 cells for bulk viral production (see section 2.6.5).

2.6.2.1 Construction of pShuttle-*CD/UPRT.*

The pShuttle vector was a different stock from that previously used but the same as that used for the CMV-GFP shuttle (Dr Katrina Sweeny, Centre for Molecular Oncology, BCI). L-VISA was digested with SalI and NotI restriction enzymes and the digested product run on a gel. The 5.7 kb expression cassette (TMPRSS2-GAL4-VP16-GAL4 binding sites-CD/UPRT) was gel extracted and ligated into the pShuttle vector (Stratagene, CA, USA), via the complementary sticky ends, using

the rapid DNA ligation kit (Roche, Germany). Briefly, 5 µl of DNA ligation buffer, 1 µl DNA ligase and 1 µl of both the vector and insert were mixed in an eppendorf and left at RT for 5 min.

1 µl of the pShuttle-CD/UPRT ligation reaction was added to 25 µl thawed chemically competent TOP10 bacteria and incubated on ice for 30 min. The TOP10 cells were transferred to a 42 ºC water bath for 30 s and then returned to ice for 2 min. 250 µl pre-warmed super optimal broth with catabolite repression (S.O.C) medium was added and the vials incubated at 37 ºC in a shaking incubator for 1 hour at 225 rpm. The bacterial culture was the plated onto agar plates containing 20 µg/ml Kanamycin. Plates were inverted and incubated overnight at 37ºC.

The following day colonies were picked and grown in 5 ml LB containing 20 µg/ml Kanamycin. The vials were placed in a shaking incubator at 37 ºC and left at 225rpm overnight. The following day 1.5 ml of each bacterial culture was miniprepped (Promega Wizard plus SV Minipreps, section 2.2.6). The miniprep DNA was digested with EcorV and BglII and run on a 0.8% agarose gel alongside both cut and uncut pShuttle DNA. Restriction digestion analysis and sequencing identified recombinant clones. All sequencing analysis was performed by the Genome Centre at Barts Cancer Institute (London) using a Sanger sequencing method and the primers listed below (Table 21).

Table 21 pShuttle-CD/UPRT sequencing primers

Primer name	Primer sequence
PshCDU 2 F	TGTCCAATTATGTCACACCACA
PshCDU 3 F	ATTCCGGCGATACAGTCAAC
PshCDU 4 F	GCTGTTCCAGTCTTTCTAGCC
PshCDU 5 F	CAGGACTGTAATATTTCCATACCA
PshCDU 6 F	GCATCCTGCTGGACTTAACC
PshCDU 7 F	CAGGGCTCAGCACCAAATA
PshCDU 8 F	TGGTGGAGGTCAGAAAGAGC
PshCDU 9 F	CTGTGAGGGAGACTGTGCAA
PshCDU 10 F	CCATCAAAACAAAACGAAACAA
PshCDU 11 F	TTCAAAGTGGGACCAGAAGG
PshCDU 12 F	AGGTGGTTGTGGTGGATGAT
PshCDU 13 F	GGGAGACTTTGGGGACAGAT
PshCDU 14 F	TATTGCCACGGCGGAACT
PshCDU 15 F	CGCTTCGAGCAGACATGATA

2.6.3 Homologous recombination

2 µg of pShuttle-CD/UPRT was linearised with Pme1 (NEB) in a 50 µl reaction volume containing 5 µl NEBuffer 4, 5 µl BSA, 1 µl Pme1, made up to 50 µl with distilled H_2O and incubated for 3 h at 37ºC. Linearised pShuttle-CD/UPRT was dephosphorylated by adding 10 µl Antarctic Phosphatase reaction buffer and 1 µl Antarctic Phosphatase and incubated at 37ºC for 15 min. The linearised and dephosphorylated pShuttle-CD/UPRT was cleaned using a standard phenol/chloroform protocol (section 2.2.3) and homologous recombination performed according to the manufacturer's instructions in BJ5183 cells (Figure 26). Briefly, linearised pShuttle-CD/UPRT was mixed together with the pAdEasy adenoviral backbone in BJ5183 cells (Addgene, USA) in a 3:1 ratio (section 2.4.8). BJ5183 are a recombination proficient bacterial strain that are deficient in certain enzymes that mediate recombination in bacteria (endA, sbcB-, recBC-, str^R), but supply the components necessary for homologous recombination to take place. Potential recombinants were grown on Kanamycin plates and sequenced using the primers listed in Table 22.

Table 22 Recombinant pAdEasy sequencing primers

Primer name	Primer sequence
Psh 1 F	GGGACTTTCCTACTTGGCAGT
Psh R	CACAATGCTTCCATCAAACG
PshCDU 2 F	TGTCCAATTATGTCACACCACA
PshCDU 3 F	ATTCCGGCGATACAGTCAAC
PshCDU 4 F	GCTGTTCCAGTCTTTCTAGCC
PshCDU 5 F	CAGGACTGTAATATTTCCATACCA
PshCDU 6 F	GCATCCTGCTGGACTTAACC
PshCDU 7 F	CAGGGCTCAGCACCAAATA
PshCDU 8 F	TGGTGGAGGTCAGAAAGAGC
PshCDU 9 F	CTGTGAGGGAGACTGTGCAA
PshCDU 10 F	CCATCAAAACAAAACGAAACAA
PshCDU 11 F	TTCAAAGTGGGACCAGAAGG
PshCDU 12 F	AGGTGGTTGTGGTGGATGAT
PshCDU 13 F	GGGAGACTTTGGGGACAGAT
PshCDU 14 F	TGTCCAATTATGTCACACCACA
PshCDU 2 F	TATTGCCACGGCGGAACT

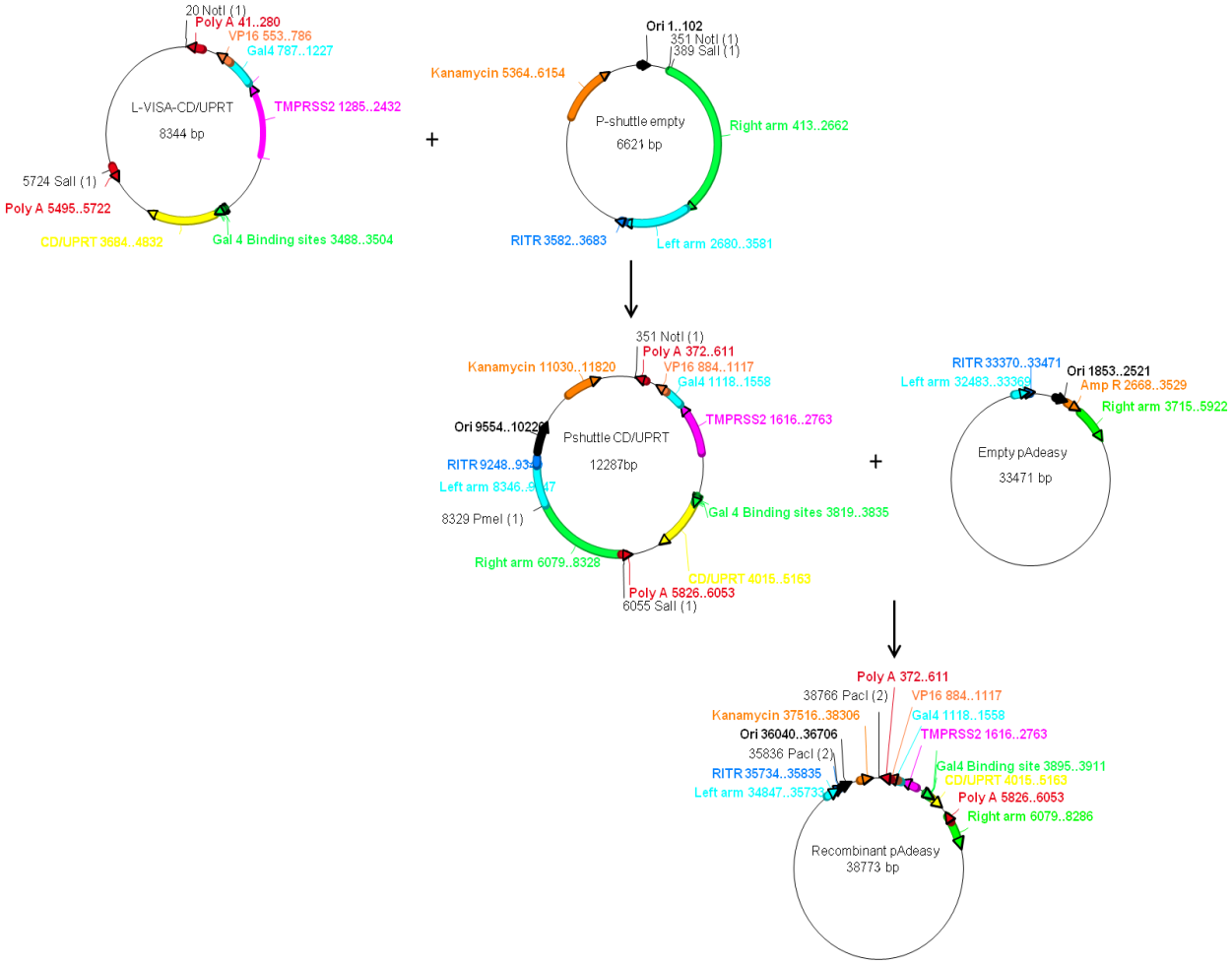


Figure 26 Diagrammatic showing homologous recombination between the pAdEasy and pShuttle vectors

TMPRSS2-VISA-CD/UPRT expression cassette was excised from L-VISA CD/UPRT and ligated into pShuttle. Homologous recombination between pShuttle and p-AdEasy resulted in the recombinant pAdEasy viral backbone containing the TMPRSS2-VISA-CD/UPRT expression cassette.

2.6.4 Generation of the novel Ad5-TV-CU.

Recombinant pAdEasy was linearised with PacI and cleaned (phenol/chloroform, section 2.4.3). 2 wells of a 6 well plate were transfected with either 0.4 µg or 0.8 µg of linearised recombinant pAdEasy (effectine, QIAGEN, Germany). Cells were incubated at 37ºC in a humidified atmosphere with 5% CO_2 and monitored for signs of cytopathic effect (CPE is determined by the presence of plaques; cells become rounded and large gaps appear between the monolayer of cells), typically between 7-10 days.

2.6.5 Large scale bulk production of virus

In order to produce a replication-defective adenovirus, the adenovirus packaging cell line HEK293 is required to provide the E1 proteins essential for assembly of infectious virus particles. Large scale bulk production of virus was performed as previously described (Wang et al. 2003). HEK293 cells are transformed with the first 4344 nucleotides (E1 gene) of Ad5, allowing them to produce adenovirus from backbone vectors without the E1 gene. Recombinant pAdEasy was linearised with Pac1, to liberate both inverted terminal repeats (ITR's) and transfected into two wells of a 6 well plate containing ~80% confluent HEK293 cells, as described in section 2.4.1). Cells transfected with recombinant pAdEasy were collected and freeze thawed three times in liquid nitrogen (N_2)(l) and heated at 37°C to release the virus from the cells. Cell debris was removed by centrifugation at 1200 rpm and media containing virus was transferred to a T75 flask containing HEK293 cells at ~80% confluency. The flask was monitored for signs of CPE, which typically occurred 48-96 h later. The cells were collected in a 50 ml falcon by gentle tapping of the flask and freeze thawed three times to release the virus. Half the viral lysate (~5 ml) was added to another T175 flask containing ~90% confluent HEK293 cells and incubated until signs of CPE occurred (typically 48-72 h). The cells were collected, freeze thawed three times, spun down at 1200 rpm to remove the cell debris and the viral supernatant split equally between 16x T175 flasks of 90% confluent HEK293. These flasks were left to incubate and monitored for signs of CPE, which typically occurred 48 h later. The cells were detached from the flasks and the media poured into two large centrifuge tubes. The tubes were spun at 200 rpm for 10 min at 4°C. The supernatant from each tube was aspirated and the pellet resuspended in 15 ml of cold PBS and transferred to a 50 ml centrifuge tube. The pellet was centrifuged for 10 min at 1000 rpm at 4°C, the supernatant aspirated and the pellet resuspended in 12 ml cold 10 mM Tris.HCL (PH 8.0). The cell suspensions were then ready for caesium chloride (CsCl) banding.

2.6.6 Caesium chloride banding

To produce a pure virus the fully encapsulated virion particles must be separated from the empty capsid particles. The standard method for purification is based on using a CsCl density gradient combined with ultracentrifugation. The viral suspension was freeze thawed three times to promote viral release and centrifuged at 6000 rpm. CsCl gradients were prepared in a 3.5" ultracentrifuge

tube (Beckman Coulter, High Wycombe, UK) by adding 10 ml 1.25 g/ml CsCl under-layered with 7.6 ml 1.4g/ml CsCl solution. The viral supernatant was layered on top and the tubes spun for 2 h at 25000 rpm at 15 °C in a Beckman SW28 swing-out rotor in an Optima LE-80K ultracentrifuge. After centrifugation 3 bands exist in the ultracentrifuge tube, a 19G needle was used to extract the bottom band that contained the encapsulated virus. The remaining bands were discarded. The encapsulated virus suspension was then split equally and layered onto 2.4 ml 1.35 g/ml CsCl solution in 4 2" ultracentrifuge tubes (Beckman) and centrifuged at 15°C, 4000rpm O/N. Again the purified virus was extracted using a 19G needle and the volume diluted 2-3 fold (up to 12 ml) with TSG buffer (96mM NaCl, 2.8mM KCl, 0.3mM $MgCl_2$, 0.5mM $CaCl_2$ and 30% (v/v) glycerol, 0.5mM Na2-HPO_4, adjusted to pH7.5). The diluted viral suspension was injected with a 19G needle into a 3-12 ml Slide-a-Lyser (Pierce Biotechnology, IL, USA) and rotated in 1L buffer containing 10 mM Tris-HCl (pH7.5), 1 mM $MgCl_2$, 150 mM NaCl and 10% (v/v) glycerol for 24 h at 4°C. The purified virus was then removed from a separate port of the dialysis chamber, aliquoted and stored at -80°C.

2.6.7 Analysis of infectability of PCa cell lines by flow cytometry (FACS).

Cells were seeded in 6-well plates at a density of $3x10^5$ (22RV1, VCaP) or $1.5x10^5$ (LNCaP) in 2 ml of their respective media. The following day cells were infected with Ad5-GFP at 10 ppc, 100 ppc, and 1000 ppc in their respective 2% media (2% FCS 1% pen/strep). Uninfected cells were used as a negative control. After 2 h the media was removed and replaced with 2 ml of their respective full media (10% FCS 1% pen/strep). Cells were incubated at 37°C for 48 h before analysis via flow cytometry. Cells were trypsinised in the wells by addition of 500 µl trypsin followed by neutralising with 500 µl 10% FCS DMEM. Suspensions were added to FACS tubes and spun at 1800 rpm for 5 min. After washing twice with 2 ml cold PBS the cell pellet was resuspended in 500 µl PBS and left on ice until acquisition. 10,000 events were acquired by flow cytometry on a FACS calibur cytometer (Becton Dickinson, Immunocytometry Systems, Belgium) with CellQuest Pro Software. The number of live events were gated using forward side scatter (FSC) versus right angle side scatter (SSC). The percentage of GFP positive cells were quantified by plotting green fluorescence (FL-1) versus FSC and setting the marker to encompass <1% of the uninfected control.

2.7 Ad5-TV-CU characterisation

2.7.1 Viral replication assay ($TCID_{50}$)

The median tissue culture infected dose ($TCID_{50}$) is defined as the amount of virus that will produce a cytopathic effect in 50% of cells. $TCID_{50}$ values were determined by seeding $1x10^4$ JH293 cells/well in a 200 µl volume in 10% FCS DMEM. The following day the purified Ad5-TV-CU virus was diluted to $1x10^{-5}$ and $1x10^{-3}$ in serum free DMEM and a previously purified and characterised Ad5wt virus was diluted to $1x10^{-7}$ as an internal control for the assay. 20 µl of the stock concentrations of both viruses were added to infect the top row of a 96-well plate, in triplicate for each virus and each stock dilution. The virus was serially diluted down the plate by transferring 20 µl from the top row into the second row and so on. The last row of the plate was left uninfected as a control. The plates were incubated for 7 to 10 days and each well scored for signs of CPE. The titre of each plate was calculated as infectious units and expressed as plaque forming units (pfu)/ml as follows:

The total number of cells exhibiting CPE was calculated per row and the following calculation used to determine the $TCID_{50}$ value.

$TCID_{50} = {}^{10\ A\text{-}D\ (S\ -\ 0.5)}$

$TCID_{50}/ml = TCID_{50} \times v$

A = Log of the highest dilution showing CPE in more than 50% of wells

D = Log of the dilution factor

S = summation of the proportion of CPE-positive wells in each row

V = infection volume = 0.02ml

pfu/ml = Log TCID50 x µ

where $\mu = -\ln p = 0.69$

According to the Poisson distribution: µ is the mean concentration of infectious particles at a given dose. p is the proportion of cultures remaining uninfected = 0.5

2.7.2 Virus particle count determination

2.7.2.1 PicoGreen

The particle count of purified virus was determined using the Quanti-iT PicoGreen dsDNA assay kit (Invitrogen), using the Tecan Infinite F200 plate reader (Tecan, Mannedorf, Switzerland) and Magellan software version 6.3 (Tecan). Viruses were heat inactivated at 56ºC for 10 min by diluting 1:2 in Tris-EDTA (TE) buffer (10 mM Tris-HCL (pH8), 1 mM EDTA) containing 0.5% SDS. After heat inactivation the viruses were serially diluted 1:6 and 1:10 in 1xTE buffer to make dilution B and C respectively. This was then later diluted 1:120 and 1:200 (Table 23). A standard curve of Lamda-phage DNA (provided in the kit) starting at 750 ng/ml was prepared using TE (Figure 27, Table 24). 100 µl of each virus or Lamda-phage DNA dilution in triplicate was transferred to a well of a 96-well plate. Picogreen reagent was diluted 200-fold in TE and 100 µl of PicoGreen reagent added to each well containing Lamda-phage DNA or virus. A linear regression curve was constructed by plotting DNA concentration vs. fluorescence (linear regression coefficient >0.98) using the fluorescent measurements from the Lambda DNA. The DNA concentration for each virus dilution was calculated using the standard curve, taking into account the dilution factor for each sample. It is assumed that 1 µg DNA is equivalent to $2.7x10^{10}$ viral particles and the number of viral particles per ml (vp/ml) was calculated based on this assumption. As an internal control Ad5wt of known particle count was included as a reference.

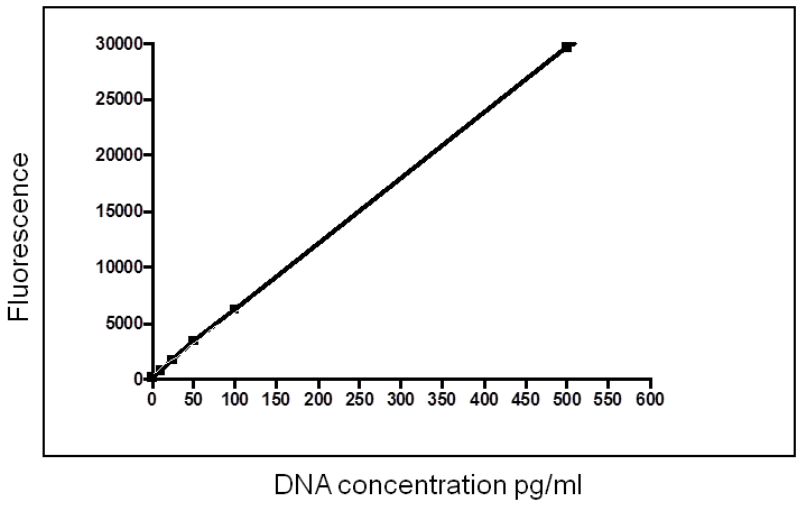


Figure 27 Picogreen lambda DNA curve

Lambda DNA was diluted as described in table 24 and a standard curve created using the Quanti-iT PicoGreen dsDNA assay kit. The standard curve was used to determine the viral particle count of Ad5-TV-CU.

Table 23 Virus dilutions for PicoGreen

Virus Sample	Volume of 1xTE/0.5% SDS (μl)	Volume of 1xTE (μl)	Volume of neat/diluted virus sample (μl)
Neat (A)	42	336	42
1:2 Diluted Virus (A)	42	357	21
1:6 Diluted Virus (B)	42	357	21
1:10 Diluted Virus (C)	42	357	21

Table 24 Control curve lambda DNA dilutions

DNA Concentration (ng/ml)	Volume of 1xTE/0.5% SDS (μl)	Volume of 1xTE (μl)	Volume of 1mg/ml Lambda DNA (μl)
750	42	63	315
500	42	168	210
100	42	336	42
50	42	357	21
25	42	367.5	10.5
10	42	373.8	4.2
1	42	377.6	0.42
0	42	378	0

2.7.2.2 Optical density

2x 100 µl aliquots of virus were removed and named VA and VB. 100 µl 2x lysis buffer was added and the viruses heated to 56 ºC for 10 min, including controls (100 µl TSG and 100 µl lysis buffer, termed CA and CB). After heating, 300 µl distilled H_2O was added to make a final volume of 500 µl. The optical density (OD_{260}) was then measured using a quartz cuvette and an eppendorf Biophotometer plus. The spectrophotometer was blanked using 500 µl dH_2O and the OD_{260} of control sample A (CA) was read, followed by two reading of virus A (VA). The machine was again blanked using 500µl distilled water and the second control sample B (CB) measured, followed by two measurements of virus B (VB). The average of the two repeat values was subtracted from the control OD_{260} reading. An average was taken of sample A and B and multiplied by the dilution factor (5). The result was divided by $9.09x10^{-13}$, giving the viral particle count (see equation below).

$$VP = \frac{mean\ OD(virus - control)x\ df}{9.09E - 13}$$

Virus particle/plaque forming units (vp/pfu) ratio was determined for each virus. A vp/pfu of <50 is desirable, however it is not possible to obtain these values for a non-replicating virus (Table 25).

Table 25 Virus particle and titre count

	Virus particle (vp) (vp/ml)	Viral titre (pfu/ml)	Vp/pfu ratio
Ad5-TV-CU #1	$2.33X10^{11}$	$3.6\ X10^{8}$	1:647
Ad5-TV-CU #2	$8.9X10^{11}$	$4.94X10^{9}$	1:180
Ad5wt	$4.63\ X10^{11}$	$5.38X10^{10}$	1:8.6
Ad5 GFP	$2.78X10^{11}$	$2.0\ X10^{8}$	1:1390

2.7.3 Confirmation of E1 and E3 deletions by PCR

2.7.3.1 Adenoviral DNA extraction

Extraction of viral DNA for use in PCR reaction was performed using QIAamp DNA Blood Mini Kit (Qiagen). 20 µl of proteinase K (Qiagen) was added to 200 µl of virus followed by 200 µl of buffer AL (supplied with the extraction kit) and incubated at 56ºC for 10 min. 200 µl of ethanol was added to the sample, vortexed for 15 sec and transferred to a QIAamp spin column within a 2 ml collection tube. The sample was centrifuged at 8000 rpm for 1 min. 500 µl buffer AW1 (supplied with the kit) was added to the tube and the sample centrifuged for 1 min at 8000 rpm. The column was placed in a new collection tube and 500 µl buffer AW2 (supplied with the kit) added to the column and centrifuged at 14000 rpm for 3 min followed by another 1 min after transfer to a new collection tube. The column was then placed in a sterile eppendorf tube and 40 µl of distilled H_2O was added to the column and centrifuged at 8000 rpm for 1 min to elute the purified DNA. The concentration of purified DNA was measured using a nanodrop spectrophotometer, DNA absorbs light at 260 nm, as the nanodrop emits light at 260 nm, the more concentrated the DNA, the less light hits the photodetector, producing a higher OD_{260}. Once the concentration was identified samples were stored at -20ºC.

2.7.3.2 Adenoviral PCR

Ad5-TV-CU used in this project was non-replicating and therefore lacked the E1 and E3 regions of the viral genome. The absence of the E1A, E1B and E3 regions were confirmed for both batches of replication-deficient Ad5-TV-CU using PCR primers specific for these regions as well as a primer pair specific to TMPRSS2 to show the presence of the promoter region (Table 26). PCR was performed using wildtype Ad5 and the pAdEasy backbone plasmid DNA as a control. PCR was set up using a standard Taq polymerase master mix (section 2.2.1). Briefly, each reaction contained 50 ng purified viral DNA, 2.5 µl each of the forward and reverse primers (10 µM), 3 µl 10x dNTPs (10 mM each), 3 µl 10x PCR buffer and 2 units (0.4 µl) Taq polymerase and made up to a final volume of 30 µl with dH_2O. DNA was amplified by 35 cycles (94°C 40s, 62°C 60s, 72°C 90 s) in a Gene Amp PCR system 9700 (Applied Biosystems; Thermo Scientific). The amplified PCR products were separated on a 1% agarose gel using a 1 kb + DNA ladder (Invitrogen) as a marker to determine the size of the PCR products (Figure 28). The expected sizes of the products were calculated from the known sizes of each sequence (Table 27) Representative gel image shown below (Figure 28).

Table 26 Adenoviral PCR primers used

Primer set	Direction	Sequence
1	5' forward	CCCGGTGAGA TTCCTCAAGAGGCCAC
	3' reverse	CCGGACCCAAGGCTCTCTGCTCTCCGGCTGCTCGGGC
2	5' forward	GTAATGTTGGCGGTGCAGGAAGGGATTG
	3' reverse	GGGTCCCCCGTATTCCTCCGGTGATAATGAC
3	5' forward	GTGTTCGCTTTGCTATATGAGGACCTGTGGC
	3' reverse	CCTCGATACATTCCACAGCCTGGCGACGCCCACC
4	5' forward	CCTGTGATTGCGTGTGTGG
	3' reverse	GACAACAGTAGCAGGCGATTC
5	5' forward	GCATCTGTGGAGAGCGGTTGTGAGACAC
	3' reverse	GCGCCAGCAGATCAAGCTCATTAGCGC
6	5' forward	GCTTAATGACCAGACACCGTCCTGAGTG
	3' reverse	GCACCAAGTGA TCGGGCCTCAGCTCC
7	5' forward	CGCTGGGGTCGCCACCCAAGATGATTAGG
	3' reverse	GAGTAGGGTACAGACCAAAGCGAGCACTG
TMPRSS2	5' forward	GGTACCAGGACAACAAGCAAAATGGC
	3' reverse	AAGCTTCAGAAGGGACAAGGGAACAA

Table 27 Binding sites of adenoviral PCR primers

Primer set	5' binding site	3' binding site	Target sequence	Expected Ad5 size (bp)
1	476	853	E1A start	377
2	767	1029	E1A inc CR2	262
3	1069	1453	E1A end	384
4	1554	2086	E1B-19K	532
5	2073	2440	E1B-55K	367
6	2383	3434	E1B-55K	1051
7	28715	29135	E3-gp19K	420
TMPRSS2	42,869,985	42,871,184	TMPRSS2 promoter	1200

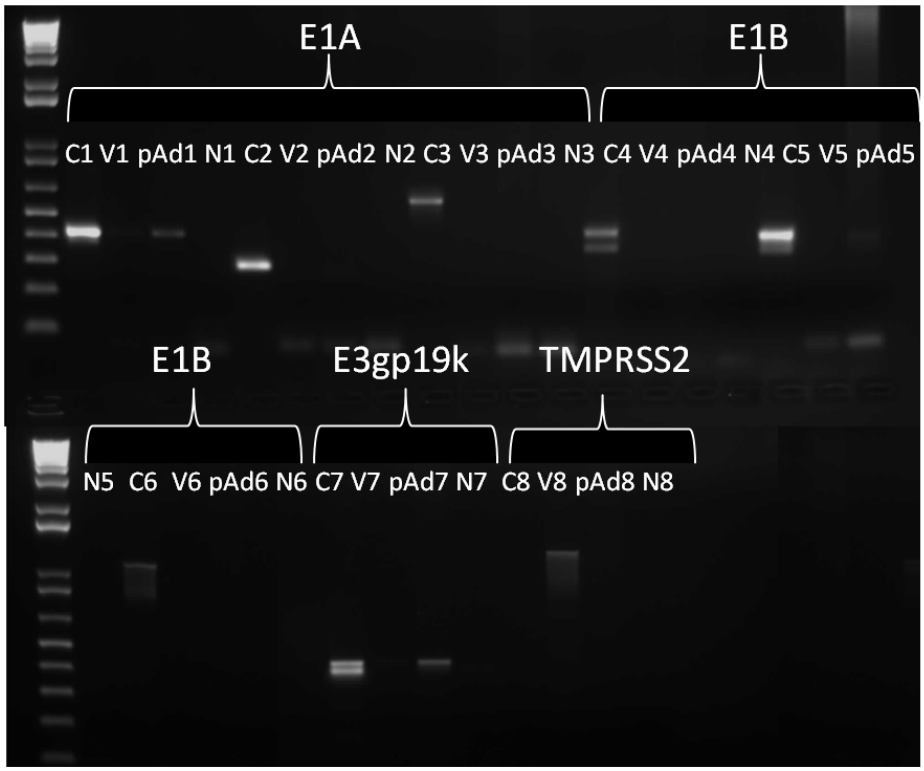


Figure 28 Adenoviral PCR

Adenoviral PCR was performed on Ad5-TV-CU to confirm the presence of the TMPRSS2 promoter and the absence of E1 and E3 regions. C= control, a wild-type replicating Ad5 V= Ad5-TV-CU, N= negative, pAd1= pAdEasy backbone which Ad5-TV-CU is based on.

2.8 Promoter activity assays

2.8.1 Luciferase assays.

Cells were seeded in 96-well plates at $2x10^4$ (22RV1, VCaP, Panc1) or $1x10^4$ (LNCaP) in 100 µl 2% respective culture media (2% FCS 0% pen/strep). The following day 0.25 µg of plasmid DNA was added to 25 µl plain media. 1 µl of lipofectamine was added to 25 µl plain respective culture media (0%FCS, 0% pen/strep). The solutions were then mixed and allowed to incubate at RT for 30 min. Following incubation 50 µl of the DNA/lipid complexes was added to the cells in the 96-well plate in triplicate. After 24/48 h 150 µl of luciferase assay reagent (Promega, USA) was added to each well on the plate and the total 300 µl was transferred to a white 96 well plate. Luminescence was read using the wallac 1420 manager plate reader.

2.8.2 Dual luciferase assays.

Cells were seeded in 96-well plates at $2x10^4$ (22rv1, VCaP, Panc1) or $1x10^4$ (LNCaP) in 100 µl respective culture media (2% FCS 0% pen/strep). The following day 20 ng of plasmid was added to 25 µl plain media as well as 2 ng of a control luciferase plasmid pRL, expressing renilla luciferase. 0.5 µl of lipofectamine was added to 25 µl plain media (0% FCS 0% pen/strep). The solutions were mixed and allowed to incubate at RT for 30 min. Following incubation, 50 µl of the DNA/lipid complexes were added to the cells in the 96-well plate in triplicate. After 24/48 h the media was removed from the cells and the cells PBS washed. 20 µl of protein lysis buffer was added to each well and left to rock gently for 20 min at RT. 5 µl of the cell lysates were added to each well of a 96-well white plate and 25 µl LARII added on top. The luminescence was read using the wallac 1420 manager plate reader. Then, 25 µl of Stop and Glo reagent (1xbuffer) was added before the renilla luciferase luminescence was read.

2.9 *In vivo*

All *in vivo* experiments were performed within the Biological Services Unit (BSU) at Queen Mary University of London under UK Home Office personal and project license authority.

Tumour growth was monitored twice weekly by calculating the volume for an ellipsoid approximated for tumour volume using the following equation:

$$Tumour\ volume = (\mathrm{LxWxWx}\pi)/6$$

Tumours were allowed to grow to a maximum area of 1.4 cm^2 or until there were signs of ulceration or poor health.

2.9.1 Establishing LNCaP-CDXR3 and LNCaP-104-S tumours xenografts in male BALB/C mice

LNCaP-CDXR3 and LNCaP-104-S cells were trypsinised and cell pellets spun down and resuspended in 10 ml of their respective culture media. Cells were counted using the BioRad cell counter system. The total number of cells required were spun down and resuspended in the required volume of cold sterile PBS. $1x10^6$ LNCaP-CDXR3 cells in 100 µl PBS were mixed with 100 µl of cold BD matrigel basement membrane complex (life science, United Kingdom) (must be kept on ice to maintain the correct consistency) and injected subcutaneously into both left and right flanks of 7 male BALB/c immunodeficient (or *nu/nu*) mice that had undergone orchiectomy (Charles River, Margate, UK). $1x10^6$ LNCaP-104-S cells in 100 µl PBS were mixed with 100 µl of cold matrigel and injected subcutaneously into each flank of 7 intact male BALB/c mice in combination with the insertion of a 1.25 mg 60-day release testosterone pellet under the skin.

2.9.2 Establishing LNCaP-104-S tumours xenografts in male NOD/SCID mice

Three 7 week old male NOD/SCID mice were injected with a 1.25 mg 60-day release testosterone pellet and three 7 week old male NOD/SCID mice were left without a pellet. 3 days later LNCaP-104-S cells were trypsinised and cell pellets spun down and resuspended in 10 ml of their respective culture media. Cells were counted using the Biorad cell counter system. The total number of cells required

were spun down and resuspended in the required volume of cold sterile PBS. $2x10^6$ LNCaP-104-S cells in 100 µl PBS were mixed with 100 µl of cold matrigel and injected subcutaneously into each flank of the 6 male NOD/SCID mice.

2.9.3 Establishing 22RV1 tumours xenografts in male CD-1 mice

22RV1 cells were trypsinised and cell pellets spun down and resuspended in 10 ml of their respective culture media. Cells were counted using the BioRad cell counter system. The total number of cells required were spun down and resuspended in the required volume of cold sterile PBS. $1x10^6$ 22RV1 cells in 100 µl PBS were mixed with 100 µl of cold matrigel and injected subcutaneously into the left flank of 12 male 5 week old CD-1 immunodeficient *nu/nu* mice the other flank was left untreated.

2.9.4 Intratumoural toxicity of Ad5-TV-CU in combination with 5-FC in BALB/c mice bearing LNCaP-CDXR3 and LNCaP-104-S xenografts

When tumours reached 100 mm^3, mice were randomly allocated to receive a single intratumoural does of $1x10^{10}$vp of either Ad5-TV-CU (n=3), Ad5-GFP (n=3) or 50 µl PBS n=6) on days 1, 3 and 5 directly into the tumour on the left flank of animals. The right flank tumour received a control dose of 50 µl PBS alone. On days 2, 4, 6 and 15 weight dependent doses (100 mg/kg) of 5-FC were administered intraperitoneally (IP) to half the mice injected with Ad5-TV-CU (n=3) and half the mice injected with Ad5-GFP (n=3) the remaining mice in the group received an equal volume control PBS injection IP. Mice were monitored for signs of ill health (loss of body weight and grooming behaviour) and tumour volume was monitored regularly for decreased growth rate. All mice were culled after 62 days and tumours harvested along with the kidneys and sent for H&E staining. Under UK Home Office regulations, mice were killed accordingly when tumours reached the maximum allowed size of 1.4 cm^2, or when there were signs of ulceration.

2.9.5 Intratumoural toxicity of Ad5-TV-CU in combination with 5-FC in CD-1 mice bearing 22RV1 xenografts

When tumours reached 100 mm^3 mice were randomly allocated to receive a single intratumoural does of 10^{10} pt of Ad5-TV-CU (n=8) on days 1, 3 and 5 directly into in the 22RV1 xenografts. The tumours on the remaining 4 mice were left

untreated. On days 2, 4 and 6 weight dependent doses (100 mg/kg) of 5-FC were administered intraperitoneally (IP) to half the mice injected with Ad5-TV-CU (n=4) the remaining mice injected with Ad5-TV-CU received an equal volume control IP PBS injection. Mice were monitored for signs of ill health and tumour volume was monitored regularly for decreased growth rate. All mice were culled by day 31 and tumours harvested for H & E staining. Under UK Home Office regulations, mice were killed accordingly when tumours reached the maximum allowed size of 1.4 cm^2, or when there were signs of ulceration.

2.9.6 Tissue preservation and processing

Harvested tumour samples and organs were prepared for paraffin processing by fixing at RT overnight in 4% formalin before transfer to 70% ethanol. Paraffin-embedded tissue sectioning and Haematoxlin and Eosin (H&E) straining were carried out by George Elia (Pathology Service, Barts Cancer Institute).

CHAPTER THREE

GENERATION OF NON-REPLICATING AD5-TMPRSS2-VISA-CD/UPRT (AD5-TV-CU) VIRUS

3.1 INTRODUCTION

Recombinant adenoviruses are useful tools for gene delivery and expression. They have been successfully used for gene therapy, vaccine therapy and in basic research (Graham and Prevec 1992, Breyer et al. 2001, McConnell and Imperiale 2004). They are capable of infecting a broad range of epithelial cell types and infection is not dependent on host cell division. Ad5 is the most commonly used human adenovirus serotype and can be rendered replication-defective by deletion of the E1A gene. The E1A gene is essential for viral replication, the E1B genes for preventing premature apoptosis and the E3 genes are responsible for evading the host immunity. Thus, deletion of all of these viral genes not only renders the virus replication-deficient, but also generates space for up to 7.5 kb foreign DNA, this is important due to the packaging limitations of the particle (Benihoud, Yeh, and Perricaudet 1999).

There are three main cloning strategies used for generating recombinant adenoviruses. These include: 1) Direct ligation of DNA sequences containing transgenes, into the adenoviral genome by complementary restriction sites. This approach is not widely used due to difficulties in finding unique restriction sites in the adenoviral genome, difficulty purifying large viral genomic DNA fragments and low ligation efficiency. 2) Utilizing site specific recombinases for example Cre recombinase/lox P that allows targeted insertion of a transgene into the adenoviral genome. 3) Homologous recombination in mammalian or bacterial cells using a two vector system. Homologous recombination requires a shuttle vector, usually containing the 5' end of the genome in which the E1 and other non essential genes are replaced with a transgene and the pAdEasy backbone vector that contains the majority of adenoviral DNA, but lacks essential genes for viral replication if making a replication-deficient virus (deleted in E1 and E3 genes). After homologous recombination the pAdEasy adenoviral backbone will contain the transgene of interest (Luo et al. 2007, Figure 29). This is a commonly used strategy for generating non-replicating adenoviruses and has been selected by me as the approach for cloning Ad5-TV-CU, as it has been used successfully to generate numerous replication-deficient viruses including several transgene-expressing non-replicating Ad5 mutants in the Halldén team (e.g. (Miranda et al.

2012, Leitner et al. 2009); Sweeney et al, manuscript in preparation) as well as in a number of other labs (Youlin et al. 2010, Ohs et al. 2013).

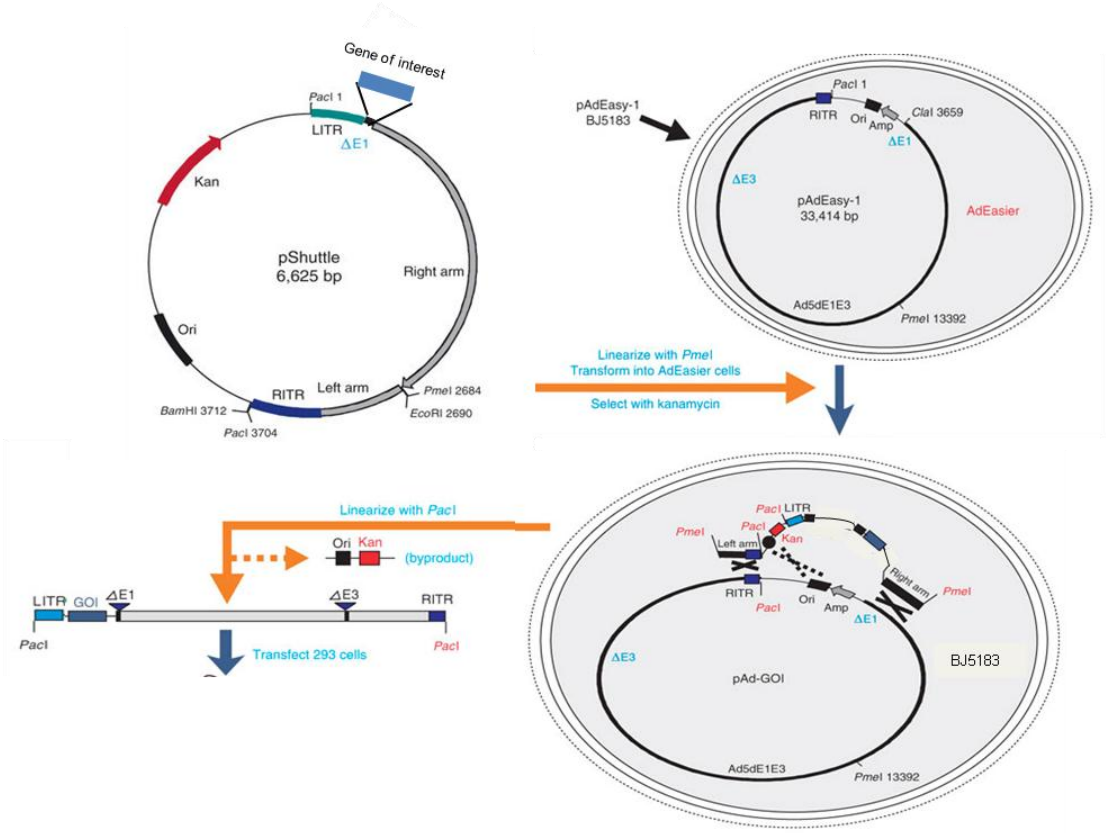


Figure 29 Schematic demonstrating the pAdEasy system.

The gene of interest is initially cloned into the empty shuttle vector and digested with Pme1. Linearised pShuttle is electroporated together with pAdEasy in BJ5183 cells to produce recombinant pAdEasy. After linearization with Pac1, recombinant pAdEasy is transfected into HEK293 cells for packaging of the virus. Adapted from (Luo et al. 2007).

For homologous recombination to take place the pShuttle vector containing the gene of interest must share regions of homology with the p-AdEasy adenoviral backbone vector. There are four different commercially available pShuttle vectors to choose from for this purpose: empty pShuttle, CMV driven pShuttle, pAdTrack and pAdTrack-CMV (Figure 30), the latter two of which contain an expression cassette encoding GFP in order to assess the transduction efficiency of the virus. Although the ability to quantify transduction efficiency is beneficial, it reduces the capacity for inserting the gene of interest, due to essential space being taken up by GFP. All four vectors share a homologous left and right arm with the pAdEasy backbone plasmid. The generation and production of all adenoviruses used in this thesis was performed in a registered biosafety level 2 laboratory.

For this study the empty pShuttle was used in order to place transgene expression (*CD/UPRT*) under the control of the TMPRSS2 promoter. Additionally, the whole expression cassette is too large to insert into a plasmid containing GFP. There are also two non-replicating pAdEasy backbone vectors to choose between: pAdEasy-1 or pAdEasy-2. pAdEasy-1 is an E1 and E3 double deleted vector, whereas pAdEasy-2 is an E1, E3 and E4 triple deleted vector. pAdEasy-1 can be easily propagated in 293 cells that express E1, however, in order to produce pAdEasy-2 derived viruses, cell lines that express both E1 and E4 are required, these are hard to come by as E4-expressing cells are often lost after serial passages. Therefore, the most commonly used backbone is pAdEasy-1, and has been chosen as is the most suitable and appropriate backbone plasmid for this study.

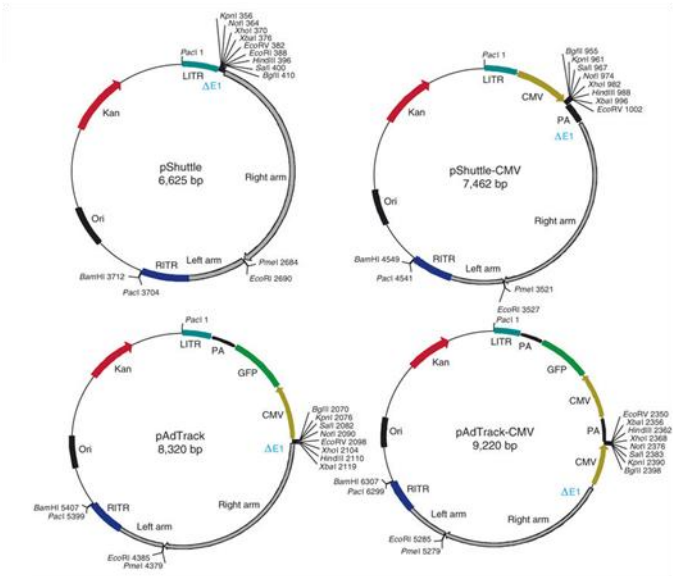


Figure 30 pShuttle plasmids available with the pAdEasy system.

There are four different shuttle plasmids available for use with the pAdEasy system empty pShuttle, CMV driven pShuttle, pAdTrack and pAdTrack-CMV, the latter two of which contain encode GFP (green) in order track viral transduction.

3.2 RESULTS

3.2.1 Generation of Ad5-TV-CU

3.2.1.1 Lack of CD/UPRT expression using a previously engineered Ad5-TV-CU adenovirus.

A replication-defective adenovirus construct had previously been generated, in which the *TMPRSS2* promoter controlled the expression of the *CD/UPRT* suicide gene by a TSTA system (see introduction section 1.7.1); constructed by Ahmet Imrali in our team, unpublished data). However, no expression of CD/UPRT after viral infection in AR-positive cell lines was detected despite potent expression from the original VISA plasmid. Steps were therefore taken to establish at which point the viral *CD/UPRT* expression had become compromised. Transfection of cells with the L-VISA vector, the original two-step-transcriptional amplification (TSTA) plasmid that has the *TMPRSS2* promoter inserted, showed good *CD/UPRT* expression (Figure 31). The expression cassette from this plasmid was inserted into a shuttle vector (pShuttle) in order to create a new Ad5-TV-CU by homologous recombination with a pAdEasy viral backbone. No expression of *CD/UPRT* was detected at protein level from pShuttle-*CD/UPRT.* The possible reasons for this were 1) the pShuttle-*CD/UPRT* plasmid is larger in size than the L-VISA plasmid and, therefore harder to transfect into cells (12kb in comparison to L-VISA which is 8kb), 2) essential elements of the expression cassette may have been removed in the cloning of pShuttle-*CD/UPRT,* 3) there may be an element in the pShuttle vector that interferes with the transcription of the *CD/UPRT* gene and 4) the orientation of the expression cassette within the pShuttle vector may affect transcription. The decision was therefore made to re-clone pShuttle-*CD/UPRT.*

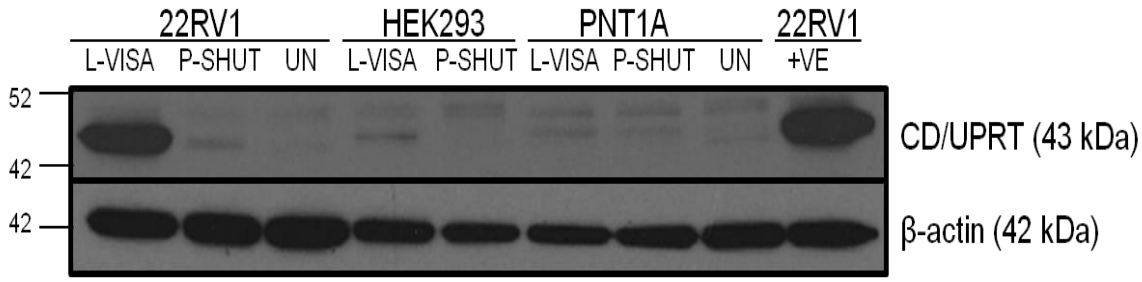


Figure 31 CD/UPRT cannot be detected after transfection with pShuttle-CD/UPRT

Western blots of protein lysates transfected with L-VISA and pShuttle-*CD/UPRT* in 22RV1, HEK293 and PNT1A cell lines. CD/UPRT protein expression (43 kDa) was visualised from L-VISA 22RV1 transfected cell lines, however only slight expression is demonstrated from pShuttle-*CD/UPRT* in 22RV1 cells. 22RV1 cell lysate previously transfected with L-VISA was used as a positive control (+ve). ß-actin was used as a loading control.

3.2.1.2 Generation of pShuttle-CMV-*EGFP*.

A pShuttle-CMV-*EGFP* control plasmid was constructed, to determine transfection efficiency of the pShuttle vector. pShuttle is required for homologous recombination with the pAdEasy adenoviral backbone (see section 2.6.3). The 1.65 kb CMV-*EGFP* construct was removed from the p*EGFP*–C2 plasmid (Dr. M. Yuan, Centre for Molecular Oncology, BCI) by digestion with AseI and MluI, blunted and ligated into an empty pShuttle vector (analogous to the CD/UPRT construct described above). Transformation of bacteria with the plasmid, selection of colonies and preparation of stock in LB Kanamycin resulted in preparation of pShuttle-CMV-*EGFP* (Figure 32). At 24 h after transfection of 22RV1 cells, more than 50% of cells were positive for GFP expression as observed by fluorescence microscopy, demonstrating that the pShuttle plasmid used in previous transgene constructions was fully functional.

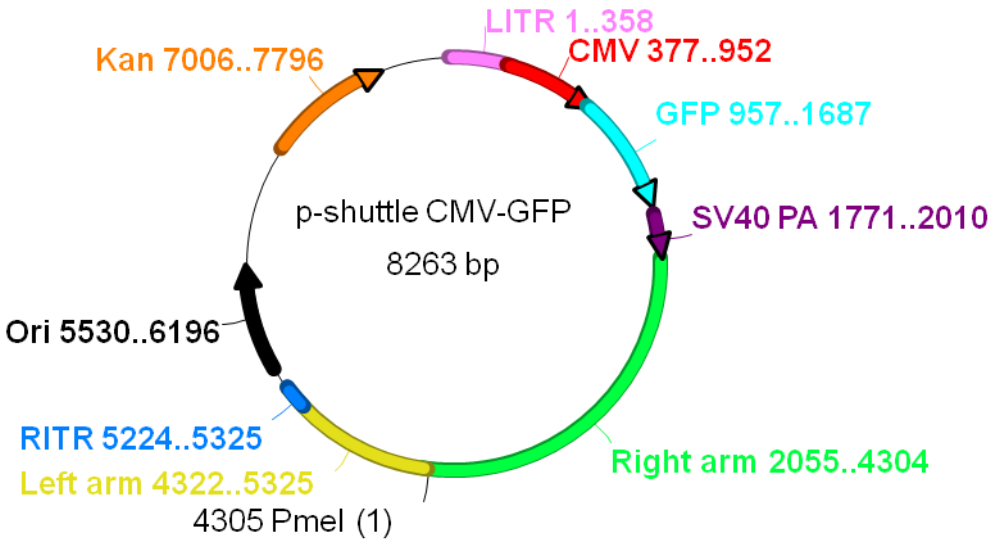


Figure 32 Plasmid map of pShuttle CMV-*EGFP*

CMV-GFP was cloned into the pShuttle vector as a control for pShuttle transfection efficiency. Activation of the universal CMV promoter drives expression of EGFP.

3.2.1.3 Generation of a new pShuttle-*CD/UPRT*.

Following demonstrable functionality of the original pShuttle the decision was made to re-clone pShuttle-CD/UPRT. Thus, the TMPRSS2-VISA-CD/UPRT expression cassette was again excised from L-VISA and cloned into an empty pShuttle vector.

7/8 of the colonies gave bands of 3.9 kb and 8.4 kb in size, corresponding to recombinant pShuttle-CD/UPRT. One colony out of eight was empty pShuttle (6 kb in size) (Figure 33A). Recombinants that showed bands at the right size after digestion were sent for sequencing (The Genome Centre, Barts Cancer Institute). Sequencing confirmed the insertion of the entire TMPRSS2-VISA-CD/UPRT expression cassette and miniprep 5 was chosen for maxiprep, resulting in preparation of pShuttle-CD/UPRT (Figure 33B).

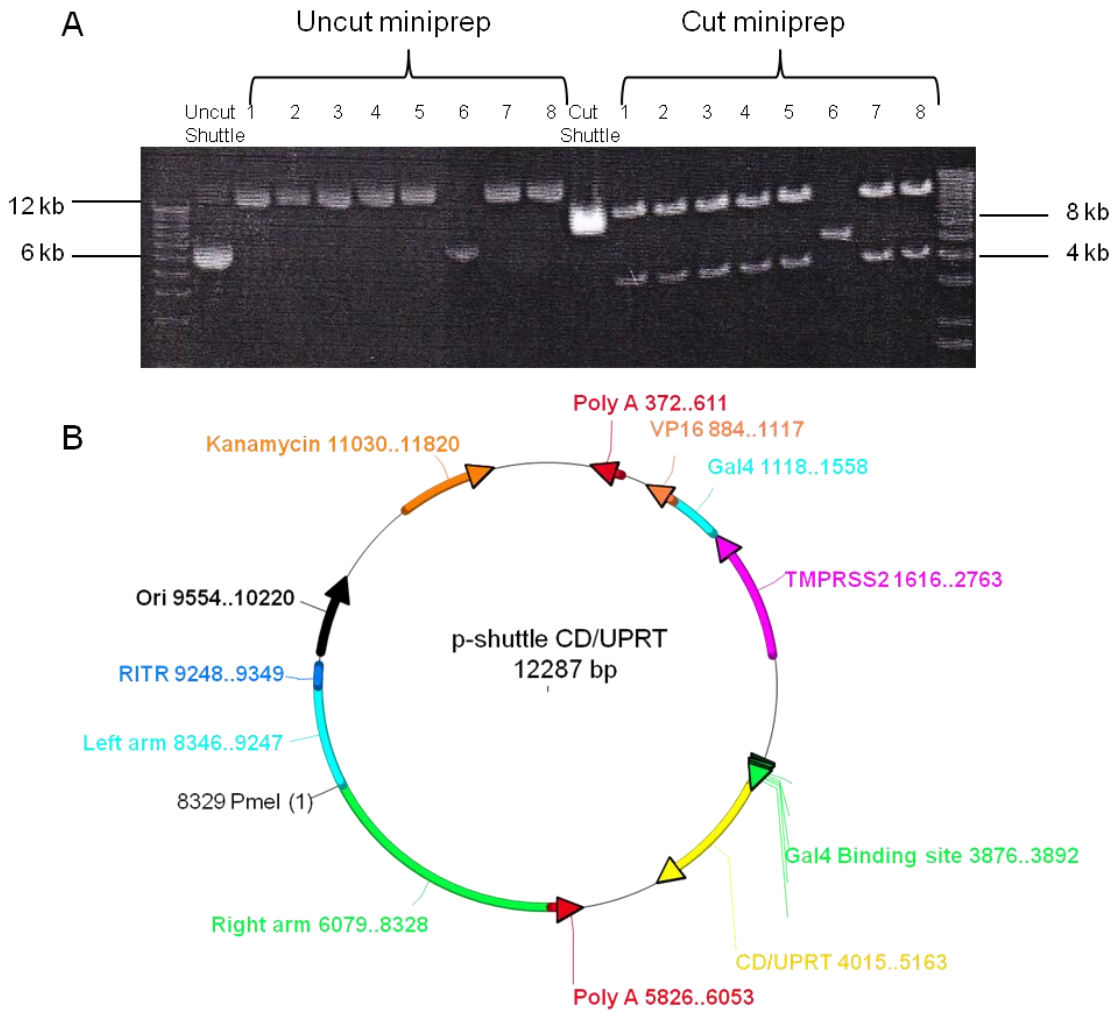


Figure 33 Plasmid map of pShuttle-TMPRSS2-VISA-CD/UPRT

A. Gel image showing 3.9 kb and 8.4 kb bands that correspond to recombinant pShuttle-CD/UPRT for eight different miniprep samples alongside the uncut plasmid. All miniprep samples except for sample 6 were recombinant. **B.** Diagrammatic showing the TMPRSS2-VISA-CD/UPRT expression cassette cloned into the pShuttle vector. The pShuttle vector has complementary left and right arms to the pAdEasy backbone, for the first step in production of Ad5-TV-CU.

3.2.1.4 CD/UPRT is more potently expressed from the L-VISA plasmid than from the pShuttle-CD/UPRT construct in 22RV1 cells.

pShuttle-CMV-*EGFP*, pShuttle-*CD/UPRT* and L-VISA were transfected into 22RV1 and HEK293 cells. pShuttle-CMV-EGFP was utilized as a control to confirm transfection efficiency, 60% of the cells showed EGFP expression at 48 h. Protein lysates were prepared 24 h and 48 h later from cells transfected with L-VISA (Figure 34A) or pShuttle-CD/UPRT (Figure 34B) and levels of CD/UPRT expression determined by Western blot. Expression levels were noticeably lower

in pShuttle-CD/UPRT transfected cells. This is likely due to the fact that the pShuttle-CD/UPRT is a much larger plasmid (12.2 kb) than L-VISA (8.3 kb) and therefore has lower transfection efficiency. This was confirmed by Quantitative-PCR (Figure 34C)

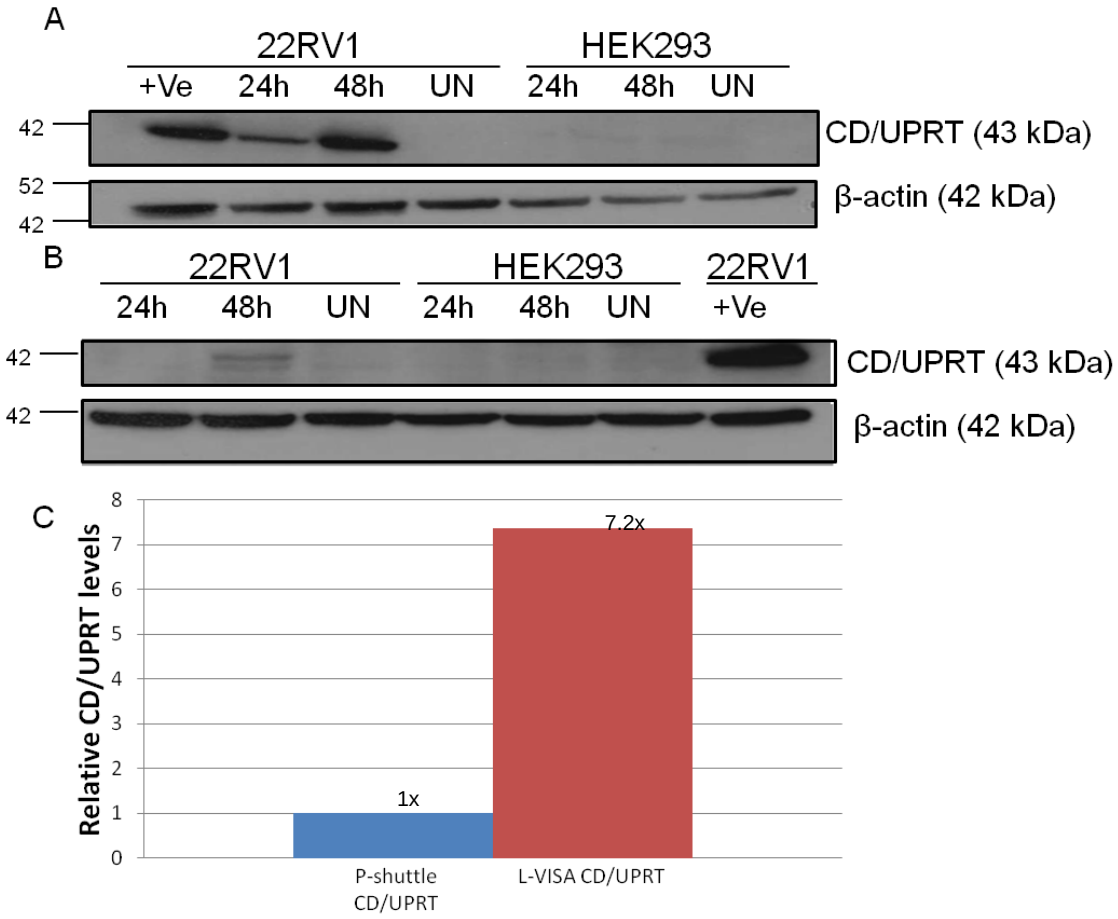


Figure 34 CD/UPRT is more potently expressed from the L-VISA plasmid than from the pShuttle-CD/UPRT construct in 22RV1 cells

Western blots of protein lysates from cells transfected with L-VISA (**A**) or pShuttle-CD/UPRT (**B**) in both 22RV1 and HEK293 cell lines. Sheep Anti-CD/UPRT Antibody was used to reveal protein expression (43 kDa). Protein expression was visualised from L-VISA 22RV1 transfected cell lines at 24 h and 48 h. However, only slight expression was demonstrated at 48 h upon transfection with pShuttle-CD/UPRT. A 22RV1 cell lysate previously transfected with L-VISA was used as a positive control (+ve). ß-actin was used as a loading control (42 kDa). Blots are representative of 3 independent experiments. **C.** Quantitative-PCR results relative to genomic DNA 48 h after transfection. One experiment with samples in duplicate, showing 7x the concentration of L-VISA plasmid entering cells compared to pShuttle- CD/UPRT.

3.2.1.5 Generation of a new recombinant pAdEasy-TMPRSS2-VISA-CD/UPRT

In order for efficient homologous recombination to take place the pAdEasy adenoviral backbone plasmid must be in its supercoiled form. A new preparation of

pAdEasy-1 was therefore made and run on a gel to determine the supercoiled status of the plasmid. The majority of the plasmid was not supercoiled after the new stock was prepared (Figure 35) but the decision was made to go ahead with the homologous recombination.

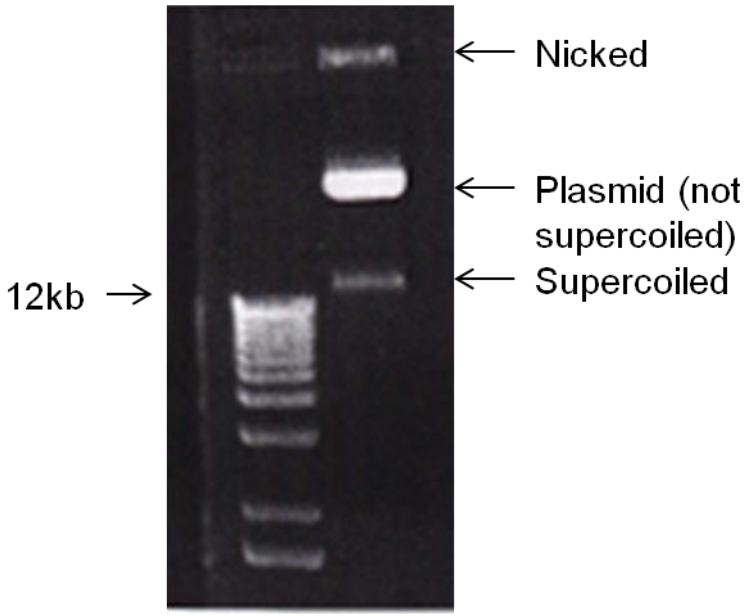


Figure 35 Generating supercoiled pAdEasy

pAdEasy was run on a 1% agarose gel to show the percentage of supercoiled plasmid, plasmid (not supercoiled) and nicked plasmid DNA. The majority of the plasmid DNA was not supercoiled (middle band), with less supercoiled (bottom band) and nicked DNA (top band).

pShuttle-*CD/UPRT* was completely linearised with Pme1 and dephosphorylated to prevent re-ligation. Homologous recombination was performed according to materials and methods section 2.6.3 and potential recombinants grown O/N on Kanamycin plates (Figure 36).

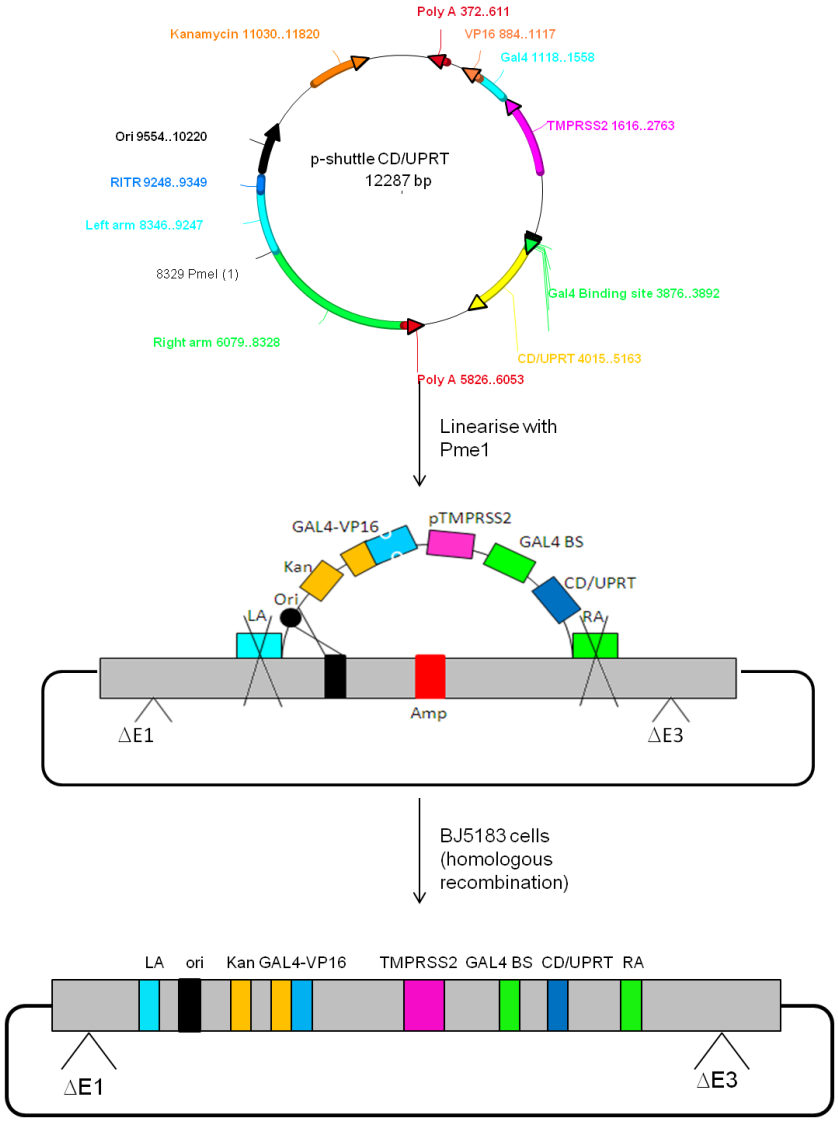


Figure 36 Diagram showing homologous recombination between pShuttle-*CD/UPRT* and the pAdEasy adenoviral backbone.

Schematic representation of the pAdEasy system. The TMPRSS2-VISA-CD/UPRT expression cassette was cloned into the pShuttle vector and linearised by digestion with PmeI. The linearised plasmid was electroporated with the pAdEasy backbone into BJ5183 cells and recombinant colonies selected for by Kanamycin resistance.

After growth on Kanamycin plates, 20 potential small recombinants (which usually represent the recombinant colonies) were selected and grown overnight in 10 ml LB containing Kanamycin. Cracking gel analysis was performed on 1 ml of bacterial culture from all 20 clones (see section 2.2.5). Four potential different populations were generated: 1) non-recombinant (pAdEasy alone 7/20), 2) non-recombinant (pShuttle-*CD/UPRT* alone 9/20), 3) recombinant pAdEasy-TMPRSS2-VISA-CD/UPRT that has undergone HR between the origin of replication (3/20) and, 4) recombinant pAdEasy-TMPRSS2-VISA-CD/UPRT that

has undergone HR between the left and right arms of the two vectors (1/20) (Figure 37).

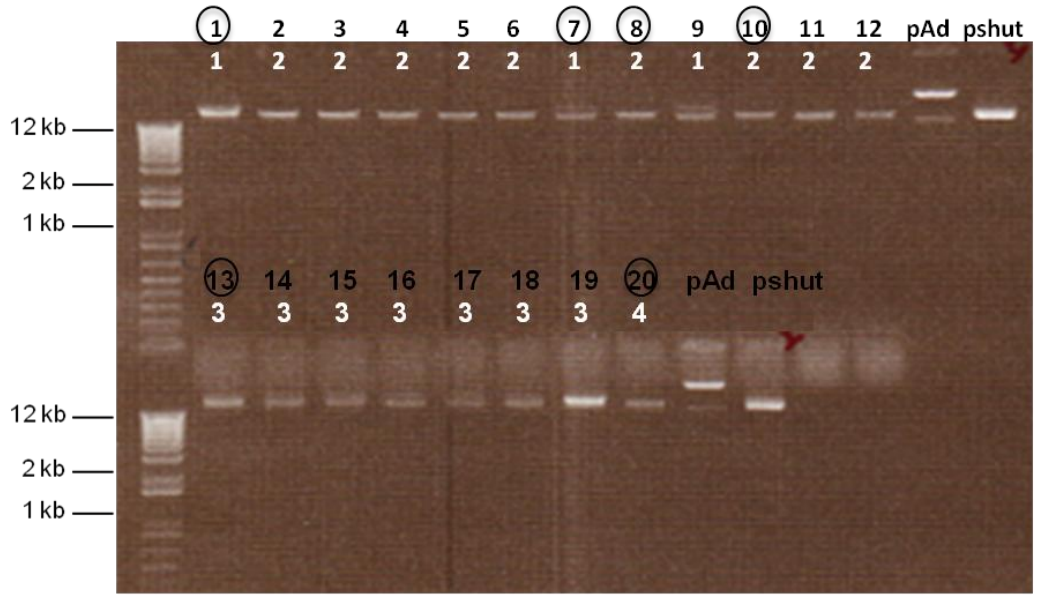


Figure 37 Cracking gel showing different clones identified after homologous recombination

20 colonies were picked and cracking gel analysis performed on 1 ml bacterial culture. Four different populations were generated (shown in white). Of the 20 colonies six were chosen for further analysis (circled) by miniprep and Pac1 digestion to confirm the four different populations.

Six of the potential recombinants were miniprepped from 1.5 ml bacterial culture and digested with Pac1 to confirm which of the recombinants contained the entire 5.7 kb TMPRSS2-VISA-CD/UPRT expression cassette (Figure 38). Digestion confirmed the four different populations that were seen on the cracking gel. Clone 20 was identified as the correct recombinant, where recombination had taken place between the left and right arms, (giving a 3 kb band plus a higher band) clones 8 + 10 (giving a 5 kb band plus a higher band) were recombinant within the origins of replication and were therefore not used any further. Clones 1 + 7 showed a 3 kb digested band and a higher band that corresponded to pAdEasy alone and finally clone 13 consisted of a 3 kb digested product and a lower band that corresponded to the pShuttle plasmid alone.

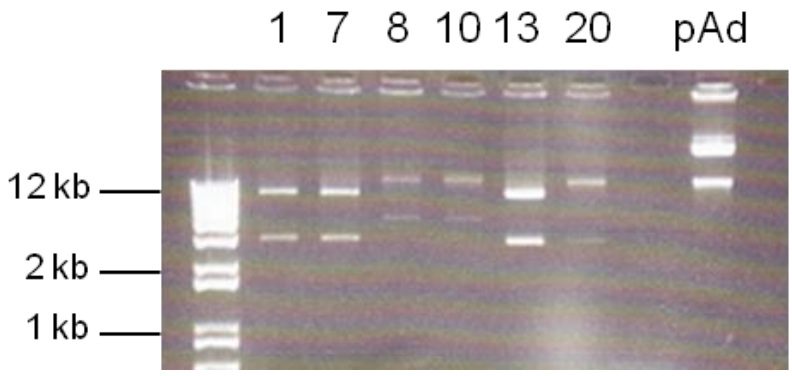


Figure 38 Restriction digest identified clone 20 as recombinant pAdEasy-TMPRSS2-VISA-CD/UPRT

Six colonies were miniprepped and digested with Pac1 to establish which clones were recombinants. Clones that were recombinant between the left and right arms generated a 3 kb band (clone 20, due to the removal of the origin of replication and the Kanamycin resistance gene). Clones that were recombinant between the origin of replication produced a slightly higher band at 5 kb (clones 8 +10) and finally clones that were pAdEasy alone produced a 3 kb band with an additional smaller band (clones 1, 7 and 13)

Clone 20 was electroporated into chemically competent Top10 bacteria, to prevent further homologous recombination within the BJ5183 bacterial cells. 3 clones were picked and digested with; Nhe1 or Pac1, all clones gave the same restriction digestion pattern and sample 20.1 was selected for maxiprep (Figure 39).

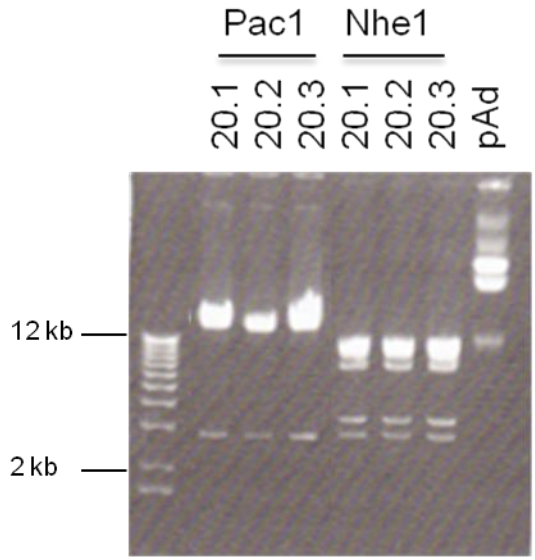


Figure 39 Restriction digest of clone 20 from electroporated TOP10 cells

All three subclones from clone 20 (20.1, 20.2 and 20.3) were digested with Pac1 to generate two bands of 3 kb and >12 kb and Nhe1 to generate multiple bands of 3168 bp, 3429 bp, 3848 bp, 8194 bp, 9100bp and 10342 bp. All three clones generated the same restriction profile.

Following identification of recombinant colonies via restriction digestion analysis, Ad5-TV-CU was produced in bulk, purified and characterised (see methods sections 2.6.5, 2.6.6 and 2.7) Caesium Chloride banding was performed to purify the virus and the correct band, corresponding to fully encapsulated Ad5-TV-CU dialysed, aliquoted into 1 ml volumes and stored at -80ºC ready for virus characterisation (Figure 40). The purified virus had a particle count of 2.33 $x10^{11}$ (Ad5-TV-CU #1) and 8.9 $x10^{11}$ (Ad5-TV-CU #2) and a viral titre of 3.6 $X10^{8}$ (Ad5-TV-CU #1) and 4.94$X10^{9}$ (Ad5-TV-CU #2).

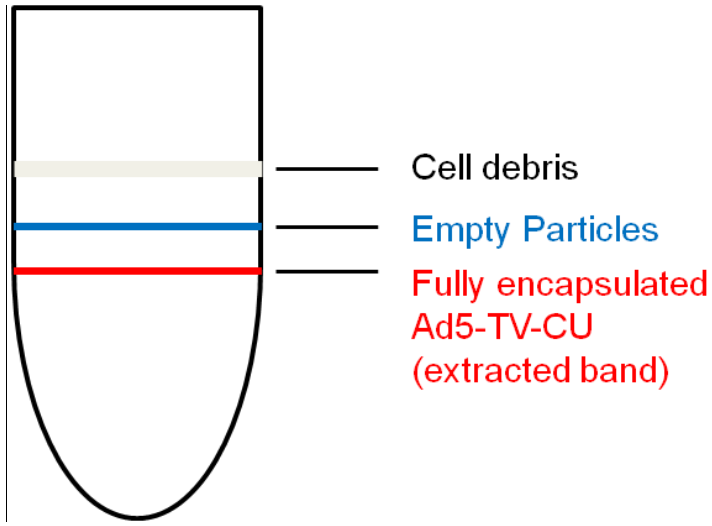


Figure 40 Caesium chloride banding

After ultracentrifugation three bands can be seen in the ultracentrifuge tube corresponding to: fully encapsulated viral particles (red) empty particles (blue) and cell debris (cream). The lower red band containing the fully encapsulated particles is extracted and dialysed.

3.3 DISCUSSION

The biggest barrier preventing the production of an effective gene therapy is the absence of a safe and efficient vector system that can transport therapeutic genes into target cells. The predominant viral vectors utilised for cancer gene therapy are currently Adenovirus, Adeno-associated virus, retrovirus and vaccinia virus, of which adenovirus is the most commonly used and best characterised in cancer. In order to produce an effective adenoviral construct steps must be taken to limit toxicity to target cells, in order to safely deliver large concentrations of adenovirus for therapeutic efficacy (Duffy et al. 2005). Targeting the activity of adenoviral gene therapy systems through use of tissue specific promoters is a good way to limit off-target toxicity. However, tissue specific promoters have not been able to generate sufficiently high levels of transgene expression to induce cell killing alone. Therefore, steps to improve transgene expression through a number of methods, including the identification of optimal promoter/enhancer regions, duplication of enhancers and promoter/enhancer fusion (Latham et al. 2000), as well as the use of two-step-transcriptional amplification systems, have resulted in increased therapeutic efficacy. In this case, Ad5-TV-CU contains the *CD/UPRT* transgene under the control of *TMPRSS2* promoter elements. In order to increase transgene expression from this promoter, a two-step-transcriptional amplification system (VP16-GAL4-WPRE integrated systemic amplifier VISA) was cloned into the vector to intensify CD/UPRT expression levels. In addition to high level gene expression, adenovirus purity is essential, as with any drug or biological product that is destined for use in humans, as it affects the potency and safety of the treatment. The production of these viruses must adhere to Good Manufacturing Processes (GMP) to even be considered for use in humans (Working, Lin, and Borellini 2005).

An original preparation of the Ad5-TV-CU virus failed to work in subsequent *in vitro* studies. There are several possible reasons for the poor activity of the original preparation of Ad5-TV-CU that could include; 1) selection of the wrong recombinant, 2) poor packaging due to the size of the TMPRSS2-CD/UPRT expression cassette, 3) failed homologous recombination and 4) an impure production of the virus. All of which led to the need to re-clone the pShuttle-*CD/UPRT* vector. Although pShuttle-*CD/UPRT* was unable to generate sufficiently

high levels of CD/UPRT expression after transfection, further investigation suggested that this was due to the poor transfectability of pShuttle-*CD/UPRT*, most probably due to its large size (section 3.2.1.4). In fact, a previous study identified difficulties in transfecting larger plasmids into primary human myoblasts (Campeau et al. 2001). PCa cells are traditionally difficult to transfect, and very few plasmid based therapies exist for PCa, therefore, in order to improve transduction of the TMPRSS2 driven CD/UPRT expression cassette, it was inserted into a non-replicating adenoviral backbone, producing Ad5-TV-CU. To accomplish this pShuttle-CD/UPRT was electroporated together with pAdEasy into BJ5183 cells to promote homologous recombination between the two plasmids and consequently produce the adenoviral backbone containing the TMPRSS2 driven CD/UPRT expression cassette. The new preparation of Ad5-TV-CU was subsequently tested *in vitro* and demonstrated CD/UPRT expression post-transfection.

CHAPTER FOUR

ACTIVITY OF AD5-TV-CU *IN VITRO*

4.1 INTRODUCTION

Prostate specific promoters have been employed to restrict transgene expression from non-replicating viruses in a number of *in vitro* studies (Latham et al. 2000, Wu, Matherly, et al. 2001), however poor transgene expression due to weak promoter activity has led to clinical failure of these viruses. Attempts to increase transgene expression have included two-step transcriptional amplification systems and chimeric promoters (Wu, Matherly, et al. 2001, Li et al. 2005). In total there have been 351 cancer adenoviral gene therapy clinical trials. Of the 351 trials, 64 have focussed on the introduction of a suicide gene to induce cell killing. Only 2 of these viral vectors have progressed to phase III clinical trials for the treatment of PCa in combination with radiotherapy and they are currently ongoing (NCT01436968 and US-0842 (Barton et al. 2008, Aguilar, Guzik, and Aguilar-Cordova 2011)). These include a virus expressing CD/HSV-TK in combination with intensity modulated radiotherapy (IMRT) and the ProstAtak virus expressing HSV-TK in combination with radiation therapy (see introduction Table 10). Neither of them utilise prostate specific promoters to drive transgene expression. Therefore, there is a need for more targeted and effective therapies for PCa.

Previous studies in our team demonstrated the strength and specificity of the 'L' promoter (in the *TMPRSS2* gene), located upstream of Exon2, driving a luciferase plasmid in 22RV1 cells (Kevin Sharp, unpublished see introduction Section 1.7.1). After cloning the L-VISA expression cassette into a pShuttle vector and subsequent homologous recombination with the pAdEasy backbone, Ad5-TV-CU was produced (Section 3.2.1.5). 22RV1 AR-positive cells demonstrated high level expression of both luciferase and CD/UPRT after plasmid transfection. Experiments were therefore performed to establish whether Ad5-TV-CU would be effective in a panel of AR-positive cell lines, including AR-positive BCa.

MM453 and MCF7 BCa cell lines belong to the apocrine subtype of BCa, with a gene profile characterized by active AR signalling. MM453 have demonstratably expressed similar levels of AR to LNCaP (Hall et al. 1994), with cell proliferation stimulated by androgens, presenting the potential for the application of Ad5-TV-CU (activated by AR stimulation of the TMPRSS2 promoter) in this cell line (Birrell et al. 1995). Some studies have also reported a role for ER in the transcriptional activation of the TMPRSS2 promoter and upregulation of the *TMPRSS2:ERG*

fusion gene in PCa as a consequence (Setlur et al. 2008, Bonkhoff and Berges 2009). This evidence highlights the cross-talk between ER and AR target genes and pathways.

It is therefore important to establish: 1) the therapeutic potential of Ad5-TV-CU in AR-positive PCa 2) the therapeutic potential of Ad5-TV-CU in AR-positive BCa and 3) whether ER is responsible for TMPRSS2 promoter activity as opposed to AR.

4.2 RESULTS

4.2.1 An initial proof of concept study in 22RV1 cells.

4.2.1.1 AR expression in PCa cell lines.

In order to establish in which cell lines the Ad5-TV-CU virus would be active, expressing the enzyme from the androgen-regulated *TMPRSS2* promoter, AR expression levels in PCa cell lines were established. AR levels in MCF7 cells, a BCa cell line that has previously been shown to express AR, were also evaluated (Guthrie et al. 1997, Cochrane et al. 2014).

Only 22RV1, VCaP and LNCaP cells expressed AR (Figure 41). 22RV1 cells showed two bands on the western blot, due to the three spliced isoforms of AR in this cell line. A full-length version with duplicated exon 3 (Ex^{3dup} at approximately 115 kDa) (Tepper et al. 2002) and two truncated versions lacking the COOH terminal domain (CTD) ($AR^{1/2/2b}$ and $AR^{1/2/3/2b}$ 70-80 kDa) (Dehm et al. 2008). These isoforms are constitutively active and similar in size, promoting expression of endogenous AR-dependent genes. VCaP cells also express two forms of AR; an amplified AR gene locus that encodes full length wildtype AR protein (110 kDa) and the shorter version of AR found in 22RV1 cells ($AR^{1/2/2b}$ 75 kDa). LNCaP cells express only the full length 110 kDa AR. The PNT2 and PNT1A normal immortalized prostate epithelial cells did not express AR protein. However, there is evidence to suggest that these cells express low levels of AR mRNA and protein (Coll-Bastus et al. 2015). In fact, AR protein expression has previously been detected in PTN2 cells with a sensitive Western blotting method (Blanchere et al. 1998) and in PNT2 and PNT1A cells by immunoprecipitation (Coll-Bastus et al. 2015). No AR protein expression could be detected in the MCF7 BCa cell line, Panc1 pancreatic cancer cell line or the primary prostate epithelial and stromal cells PrEC and PrSC.

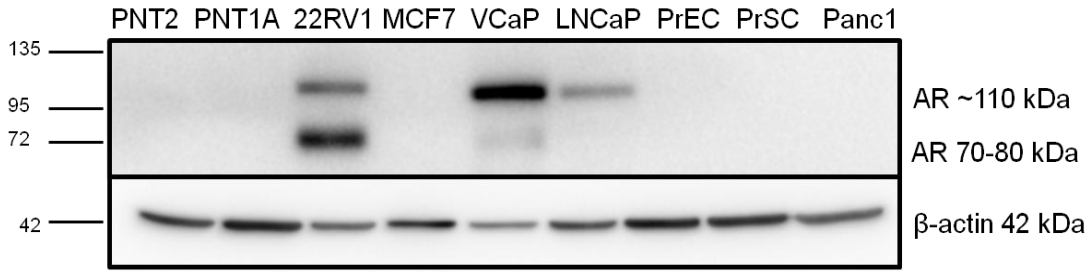


Figure 41 AR expression in seven PCa cell lines, the MCF7 BCa cells and the Panc1 pancreatic cancer cells

Cell lysates were collected and 30 µg loaded on a protein gel. Rabbit anti-AR antibody was used to reveal AR expression in all cell lines. AR was only detected in 22RV1, VCaP and LNCaP as expected. Mouse anti-ß-actin antibody was used as a loading control (42 kDa).

4.2.1.2 CD/UPRT protein expression was detected in 22RV1 cells following viral infection.

To verify the activity of Ad5-TV-CU in AR-positive cell lines and evaluate CD/UPRT transgene expression, 22RV1 AR-positive, androgen-independent cells, were used for an initial proof of concept study. *TMPRSS2* is an androgen-regulated gene. It is therefore expected the promoter of *TMPRSS2* is activated by binding of AR to one or several of the identified AREs in the 5' UTR of the gene. Therefore, we expected that the Ad5-TV-CU virus would express the CD/UPRT protein in cells with a functional AR (22RV1, VCaP and LNCaP) and not in AR-negative cells (DU145, HEK293, Panc1). A dose dependent increase in *CD/UPRT* expression (43 kDa) was detected in 22RV1 cells, while the AR-negative DU145 cells showed no expression of CD/UPRT, demonstrating that CD/UPRT expression from Ad5-TV-CU is specific to AR-expressing cells (Figure 42).

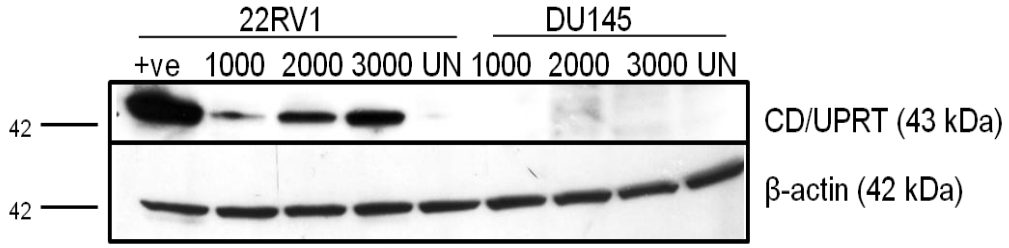


Figure 42 CD/UPRT protein expression was detected in 22RV1 cells but not DU145 cells following viral infection

22RV1 and DU145 cells were infected with 1000 ppc, 2000 ppc and 3000 ppc of Ad5-TV-CU and protein lysates collected at 48 h. 30 µg lysate was run on a protein gel. A 22RV1 cell lysate previously transfected with L-VISA was used as a positive control (+ve). Sheep anti-CD/UPRT antibody was used to reveal CD/UPRT expression (43 kDa). Mouse anti-ß-actin antibody was used as a loading control (42 kDa). Representative blot of 5 independent experiments.

4.2.1.3 CD/UPRT protein expression did not increase in 22RV1 cells upon addition of Mibolerone nor decrease upon addition of Bicalutamide.

It was important to establish whether the levels of CD/UPRT expression could be increased upon addition of the synthetic androgen Mibolerone, or decreased by the anti-androgen Bicalutamide due to AR dependent transcriptional activation of Ad5-TV-CU in 22RV1 cells. To establish whether the levels of *CD/UPRT* expression could be increased by activation of AR, the AR agonist mibolerone was added to 22RV1 cells after infection with Ad5-TV-CU at 1000 and 2000 ppc. No apparent increase in CD/UPRT protein expression was detected with the addition of 1 nM mibolerone at 48 h or 72 h. Western blot showing transgene expression at 48 h is shown in Figure 43A.

To test whether the mutated AR expressed in 22RV1 cells is also less responsive to antagonism, the AR antagonist Bicalutamide was added to cells after infection with Ad5-TV-CU and levels of CD/UPRT transgene assesed at 48 and 72 h. No apparent decrease in CD/UPRT protein expression was detected after infection with Ad5-TV-CU at 1000 and 2000 ppc in combination with 5 µM Bicalutamide. Western blot showing transgene expression is shown in Figure 43B. These data suggest a potential new treatment strategy for PCa that simultaneously targets PCa specific cell death through Ad5-TV-CU, whilst limiting PCa cell growth through Bicalutamide, with no effect on Ad5-TV-CU efficacy. This would need to be further explored in a number of AR dependent cell lines to establish the true clinical benefit of this concomitant therapy.

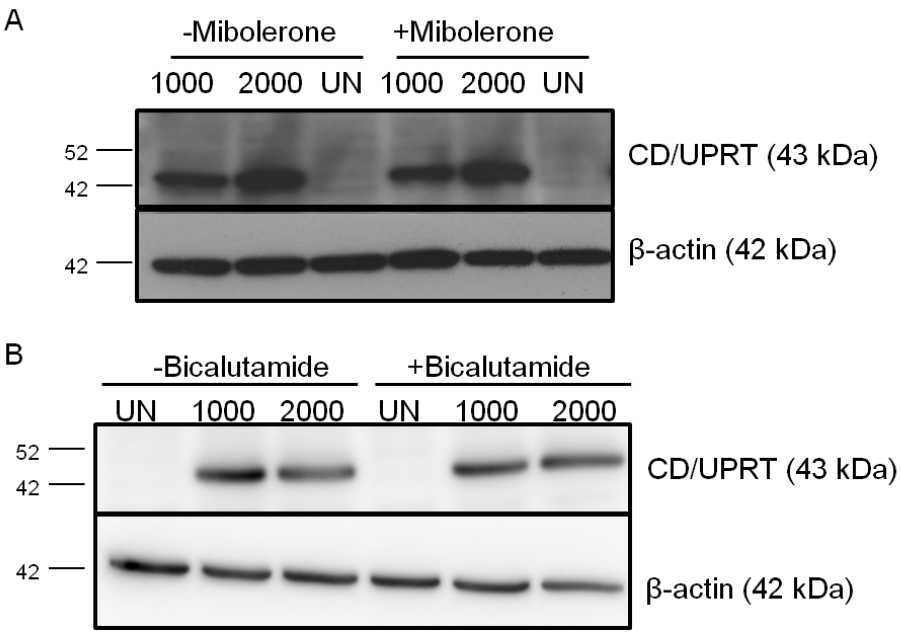


Figure 43 CD/UPRT protein expression did not increase in 22RV1 cells upon addition of synthetic androgen mibolerone, nor decrease upon addition of anti-androgen Bicalutamide.

22RV1 cells were infected with 1000 and 2000 ppc of Ad5-TV-CU. Two hours after infection the media was removed and replaced with 10% C/S media containing **A.** mibolerone (1 nM) or **B.** Bicalutamide (5 µM). Cells were lysed after 48 h and 30 µg of protein run on a gel, UN= uninfected. Sheep Anti-CD antibody was used to reveal CD/UPRT expression (43 kDa). Mouse anti-ß-actin antibody was used as a loading control (42 kDa). Representative blots of 3 independent experiments.

4.2.1.4 Addition of the non-toxic prodrug 5-FC to 22RV1 cells infected with Ad5-TV-CU, results in dose-dependent cell killing.

It was important to establish the specificity and toxicity of the replication-defective adenoviral mutant (Ad5-TV-CU) alone in 22RV1 cells, to establish the potency of the virus in 22RV1 AR-positive cells in comparison to other AR-positive and AR-negative PCa cell lines. Dose response curves were generated and cell death assessed three days post-infection to determine viral EC_{50} (Effective Concentration, the dose required to kill 50% of cells) values. One representative graph out of 5 independent experiments is shown in Figure 44A and the mean EC_{50} value for these experiments was 485 ppc ± 96.7 ppc.

Following on from this, the toxicity of 5-FC (pro-drug) and 5-FU (toxic metabolite) were determined to establish an appropriate concentration at which to add 5-FC in combination with Ad5-TV-CU. Dose response curves were generated for both drugs in 22RV1 cells from the highest feasible dose (dependent on stock concentration) of 1 mg/ml (Figure 44B). 5-FC alone is non-toxic to 22RV1 cells up

to 500 µg/ml, however the highest feasible dose of 1 mg/ml resulted in 35-45% cell death. As expected 5-FU is highly toxic to 22RV1 cells, the average EC_{50} value from five independent experiments was 1.75±0.42 µg/ml. These results confirm that 22RV1 cells can tolerate doses of 5-FC up to 500 µg/ml before cell killing is initiated, doses below 500 µg/ml are therefore defined as suboptimal. However, doses of 1.75 µg/ml 5-FU are sufficient to kill 50% of cells, confirming that only a very small quantity of 5-FU, converted from 5-FC by CD/UPRT, is required to induce high levels of cell killing. Therefore, the optimal dose of 500 µg/ml 5-FC in combination with Ad5-TV-CU will induce the maximum levels of 5-FU specific cell death.

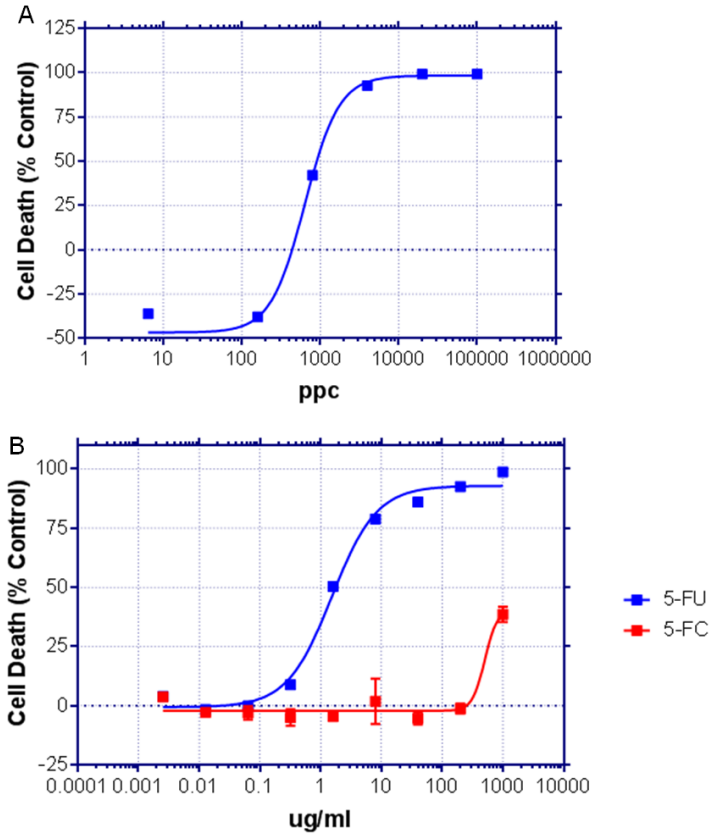


Figure 44 Dose-response assays to establish EC_{50} values for Ad5-TV-CU, 5-FC and 5-FU in 22RV1 cells.

A. Ad5-TV-CU was diluted 1/5 from $1x10^5$ to 0.2 ppc. 10 µl of the viral dilution was added to 90 µl or media containing $2x10^4$ 22RV1 cells in each 96-well. MTS was performed after 72 h and cell viability calculated relative to untreated control wells. The mean EC_{50} was 485±96.7 ppc, n=5. **B.** 5-FU and 5-FC drugs were diluted 1/5 from 1 mg/ml to 2.5 ng/ml, 10 µl of the dilutions were added to 90 µl of media containing $2x10^4$ 22RV1 cells in a 96 well plate. MTS was performed after 72 h and cell viability calculated relative to untreated control wells. It was not possible to establish a dose response curve with 5-FC, while the addition of 5-FU resulted in an average EC_{50} of 1.75 ± 0.42 µg/ml, n=5. Data shown is mean of duplicates ± SEM from one experiment, representative of five.

Once the EC_{50} values for Ad5-TV-CU, 5-FC and 5-FU alone had been generated, 5-FC was combined with Ad5-TV-CU in prodrug sensitization assays to demonstrate enhanced and AR-specific cell killing, due to the target cell selectivity of Ad5-TV-CU. Doses of 5-FC were selected from the dose-responses to 5-FU covering a range of concentrations (1-500 µg/ml). None of the selected concentrations of 5-FC induced toxicity in 22RV1 cells alone, apart from the highest concentration of 500 ug/ml, that induced 45% cell death alone (Figure 44B). Cell viability was calculated relative to untreated control well for virus alone, or cells treated with drug alone for combination of Ad5-TV-CU and 5-FC. The combination of Ad5-TV-CU and increasing concentrations of 5-FC resulted in 5-FC dependent increased cytotoxicity (Figure 45A), illustrated by decreased viral EC_{50} values in response to increasing doses of 5-FC (Figure 45B). Increased cytotoxicity was accomplished with all doses of 5-FC, except for the lowest dose of 1 µg/ml, that had no effect on virus-mediated cell killing. There was a significant decrease in EC_{50} values with the combination of Ad5-TV-CU and 100 µg/ml 5-FC in comparison to Ad5-TV-CU alone (p-value <0.05). This difference became highly significant when 500 µg/ml 5-FC was added in combination with Ad5-TV-CU (p-value <0.01). Representative data from four independent experiments is shown (Figure 45B). Average EC_{50} values ± SEM of virus alone and in combination with 5-FC can be seen (Table 28).

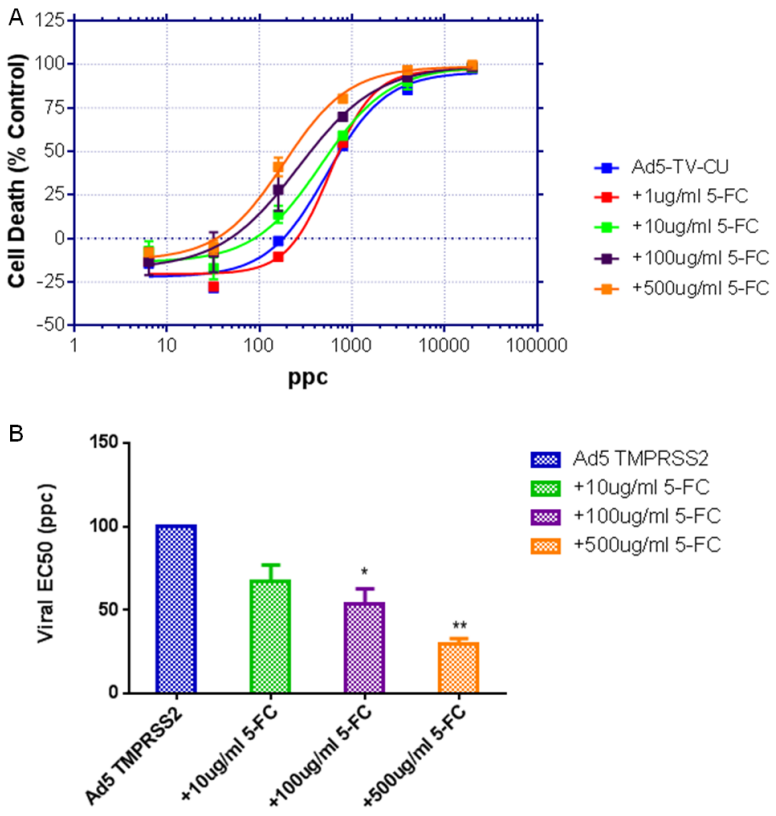


Figure 45 Combination of Ad5-TV-CU and 5-FC in 22RV1 cells generates 5-FC dose dependent shifts in EC_{50} values

A. Ad5-TV-CU was diluted 1/5 from $1x10^5$ ppc to 0.2 ppc. 10 µl of the viral dilution was added to 80 µl (for the combination with 5-FC or 90 µl for virus alone) of media containing $2x10^4$ 22RV1 cells/well. Fixed doses of 5-FC were added at 1 µg/ml, 10 µg/ml, 100 µg/ml and 500 µg/ml and MTS performed after three days. Cell viability was calculated relative to untreated control wells for virus alone, or cells treated with drug alone for combination of Ad5-TV-CU and 5-FC. Data shown is mean of duplicates ± SEM from one experiment, representative of four. **B.** Graph shows reduction in EC_{50} values in 22RV1 cells treated with fixed 5-FC prodrug at 10 µg/ml, 100 µg/ml and 500 µg/ml. Statistical analysis was performed using the 1 way ANOVA test with Tukey's multiple comparison test. * p-value <0.05, ** p-value <0.01, n=4.

Table 28 Average EC_{50} values for combinations of Ad5-TV-CU and 5-FC ± SEM in 22RV1 cells

TREATMENT Ad5-TV-CU dose response + 5-FC (**µg/ml**)	Ad5-TV-CU **EC_{50}** values (ppc)
0	485.4 ± 96.7
1 µg/ml	560.2 ± 118.8
10 µg/ml	327.6 ± 63.1
100 µg/ml	265.2 ± 52.8 *
500 µg/ml	145.0 ± 31.1 **

*** p-value <0.05, ** p-value <0.01**

4.2.2 Activity of Ad5-TV-CU in other AR-positive cell lines.

4.2.2.1 No 5-FC dose dependent cytoxicity is observed in prodrug sensitization assays with AR-positive, androgen-dependent cell lines LNCaP and VCaP

Whilst the enhanced cell killing in 22RV1 cells was encouraging, it was important to also validate these results in a larger panel of AR-positive cell lines to determine the AR specific transcriptional activation of Ad5-TV-CU. To fit this purpose the same experiments were conducted in two additional AR-positive cell lines, LNCaP and VCaP. Dose-dependent increases in CD/UPRT expression were observed following infection of VCaP cells with Ad5-TV-CU at 1000, 2000 and 3000 ppc (Figure 46A). However, only small dose-dependent increases in CD/UPRT expression could be detected in LNCaP cells following Ad5-TV-CU infection, that could not be replicated in two further experimental repeats (representative blot in Figure 46B). The doses of Ad5-TV-CU selected to infect LNCaP cells were much higher than 22RV1 and VCaP AR-positive cell lines, yet were unable to induce CD/UPRT transgene expression. Possible reasons for this may be that LNCaP cells are less infectable than 22RV1 and VCaP, or that they are not capable of stimulating AR-dependent transcriptional activation of Ad5-TV-CU (discussed below).

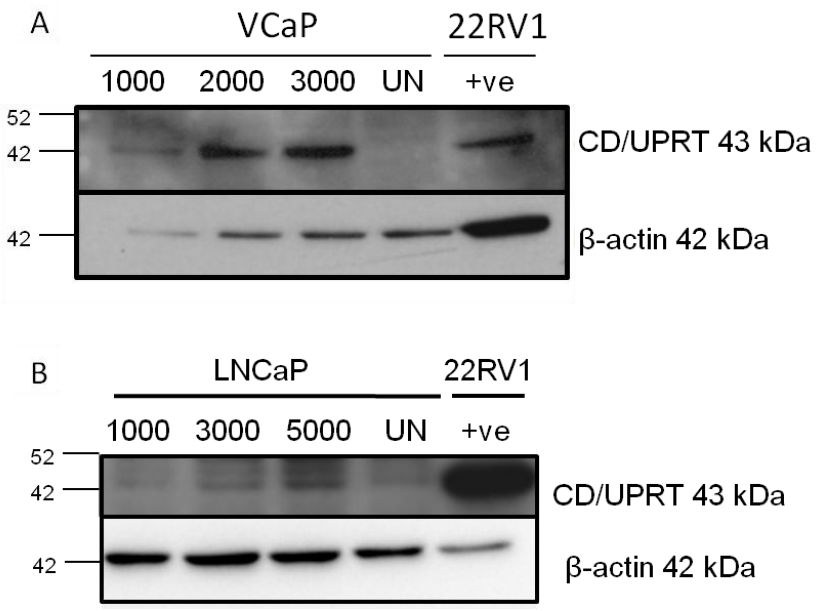


Figure 46 CD/UPRT protein expression was detected at high levels in VCaP cells but not LNCaP cells following Ad5-TV-CU viral infection

A. VCaP cells were infected with Ad5-TV-CU at 1000 ppc, 2000 ppc and 3000 ppc. Protein lysates were collected at 48 h and 30 µg of protein run on a gel. A dose dependent increase in CD/UPRT expression was detected. Representative blot of two independent experiments. **B.** LNCaP cells were infected with Ad5-TV-CU at 1000 ppc 3000 ppc and 5000 ppc. Protein lysates were collected at 48 h and 30 µg protein/lane was separated on a gel. Low levels of dose-dependent CD/UPRT expression were detected in one experimental repeat. Representative blot from three independent experiments. Previously infected 22RV1 cells were used as a positive control for CD/UPRT expression. Sheep Anti-CD antibody was used to reveal CD/UPRT expression (43 kDa). Mouse anti-ß-actin antibody was used as a loading control (42 kDa).

As described in section 4.2.1.4, it was important to establish the toxicity of 5-FC (pro-drug) and 5-FU (toxic metabolite) in the two additional AR-positive cell lines, LNCaP and VCaP in order to establish an appropriate dose of 5-FC for prodrug sensitization assays. Dose-response curves to 5-FC and 5-FU were generated in LNCaP and VCaP cells (Figure 47A+47C). 5-FC was non-toxic to LNCaP cells up to 500 µg/ml, similar to the toxicity seen in 22RV1 cells. The highest feasible dose of 5-FC at 1 mg/ml resulted in <50% cell death in LNCaP cells. LNCaP cells were also highly sensitive to the toxic metabolite 5-FU, low doses of 1.3 µg/ml were sufficient to kill 50% of the cells (Figure 47A), suggesting that very low levels of 5-FU could induce high levels of LNCaP cell death. VCaP cells were resistant to 5-FC, with no detectable cell killing at any dose. VCaP cells also appeared more resistant to 5-FU than LNCaP cells and cell death reached only 75% with treatment at the highest feasible dose of 5-FU (1 mg/ml) (Figure 47C), indicating

that higher doses of 5-FC would be required to generate sufficiently high levels of cytotoxic 5-FU in VCaP cells compared to 22RV1 and LNCaP cells.

Unsurprisingly, there were no shifts observed in dose-response curves, and therefore no changes in EC_{50}-values, in the AR-positive LNCaP cells upon addition of 5-FC in combination with Ad5-TV-CU (Figure 47B). This was expected, since CD/UPRT protein was only detected at very low levels post-infection in one experimental repeat (Figure 46B). Moreover, no additional cell killing was observed in VCaP cells, which expressed high levels of CD/UPRT after infection (Figure 47E + 47F). This is likely due to the high insensitivity of the cells to both 5-FC and 5-FU (Figure 47D). Therefore, even if CD/UPRT is expressed and can convert 5-FC to 5-FU in VCaP cells, the levels are not sufficient to cause significant cell killing. A possible reason for this insensitivity might be that VCaP cells have a long doubling time (5-6 days) and 5-FUdMP causes cell death by thymidylate synthase inhibition and incorporation into the DNA and RNA, resulting in lethal DNA and RNA damage that requires cells to enter S-phase (see introduction section 1.5). Therefore, three days may not be long enough to generate sufficient cytotoxicity in cells with slow passage through the cell cycle, resulting in minimal cell death through 5-FU treatment (discussed further below, Figure 49).

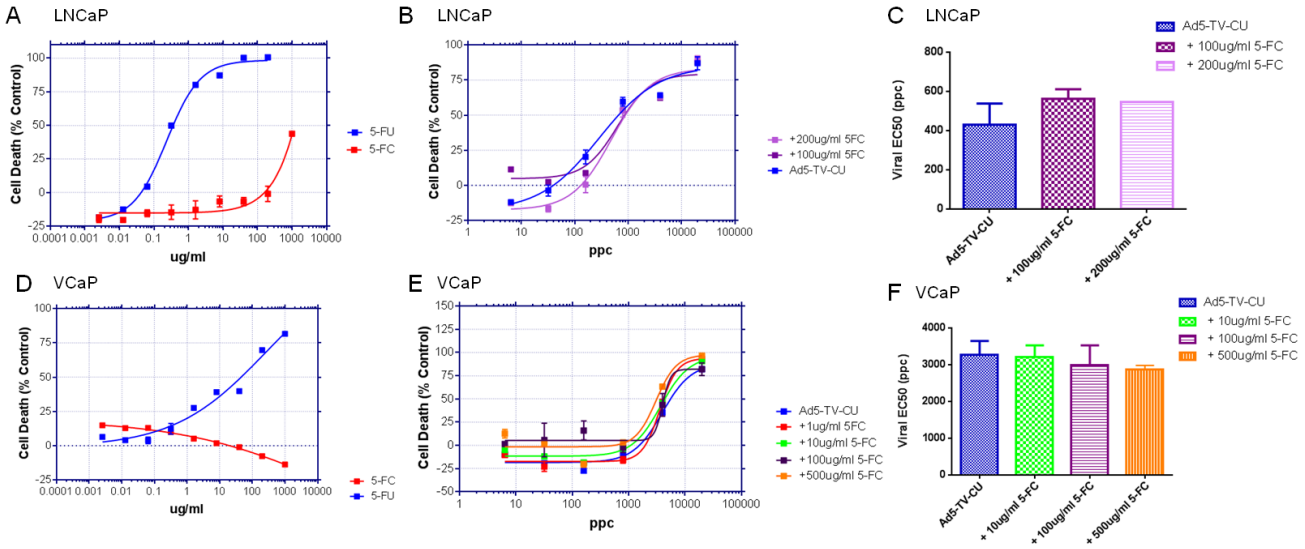


Figure 47 Combination of Ad5-TV- CU and 5-FC in VCaP and LNCaP cells does not generate significant 5-FC dose-dependent decreases in EC_{50}-values

A + D 5-FC and 5-FU were titrated 1/5 across a 96 well plate from a starting concentration of 1 mg/ml to 2.5 ng/ml. 10 µl of the titration was added to 90 µl of media containing $1x10^4$ LNCaP or $2x10^4$ VCaP cells. It was not possible to establish a dose response curve for LNCaP or VCaP cells administered with 5-FC. However, EC_{50} in LNCaP cells administered with 5-FU was 1.3 µg/ml and the highest feasible dose of 5-FU (1 mg/ml) killed only 75% of VCaP cells. **B + E** Ad5-TV-CU was titrated 1/5 across a 96 well plate and 10 µl added to 80 µl (or 90 µl for virus alone) of media containing $1x10^4$ LNCaP or $2x10^4$ VCaP cells. Fixed concentrations of 100 µg/ml and 200 µg/ml (LNCaP) or 1 µg/ml, 10 µg/ml, 100 µg/ml and 500 µg/ml (VCaP) 5-FC were added. There was no shift in dose response observed upon addition of the 5-FC prodrug in either cell line. Data shown is mean of duplicates ± SEM from one experiment representative of 2. **C + F** Bar charts show no significant decrease in average EC_{50} ± SEM in LNCaP or VCaP cells infected with Ad5-TV-CU in combination with increasing doses of 5-FC. MTS assays were performed after 3 days and cell killing calculated relative to untreated control wells or wells containing drug alone (for combination) (n=2).

In order to rule out poor infectability as the cause of limited CD/UPRT transgene expression in LNCaP cells after Ad5-TV-CU infection, and to establish the infectability of a wide range of prostate cancer cell lines, LNCaP-104-S, LNCAP-104R1, LNCaP-CDXR3, HEK293, Panc1, 22RV1, VCaP and LNCaP cells were assessed using a non-replicating Ad5-GFP virus. All cell lines were infected with 100 ppc Ad5-GFP and the % of GFP positive cells analysed by flow cytometry. All three of the LNCaP sublines (LNCaP-104-S, LNCaP-104R1 and LNCaP-CDXR3) demonstrated similar infectability (~25%), whilst the LNCaP subline in our group (ATCC) had a much higher percentage of GFP positive cells (~48%). There were only 10% more infected 22RV1 cells than LNCaP, and a similar percentage of VCaP cells to 22RV1 cells were positive for GFP, suggesting that all three AR-positive cell lines used for preliminary studies had similar infectability (Figure 48). Therefore, this does not explain the lack of CD/UPRT expression post infection in LNCaP (ATCC).

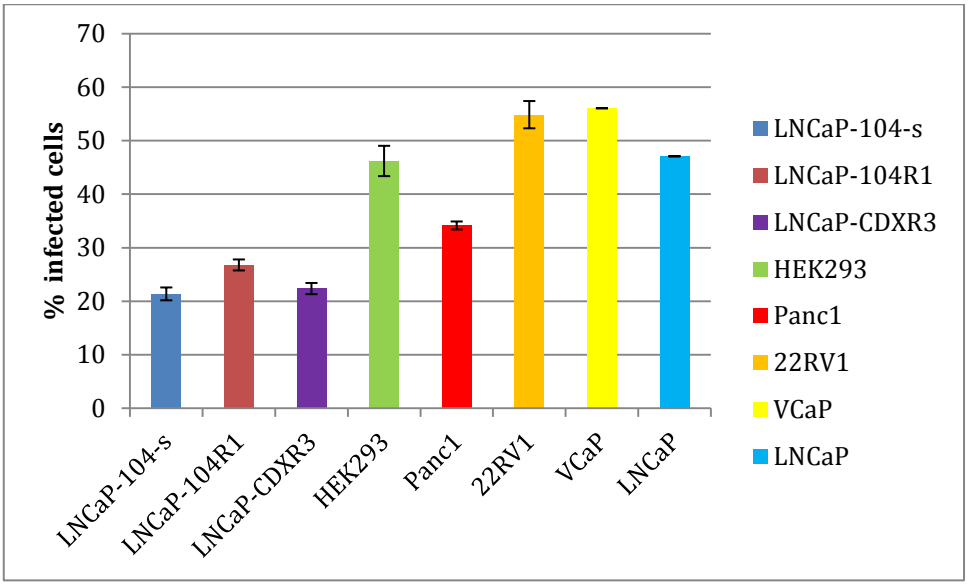


Figure 48 Infectability of AR-positive PCa cell lines

Cells were seeded in 6 well plates and the following day infected with non-replicating Ad5-GFP at 100 ppc. After 2 h the media was removed and replaced with 10% DMEM. At 48 h post infection, cells were harvested for flow cytometry analysis. Results shown are mean of duplicates from one experiment.

To establish if resistance to 5-FU in VCaP cells is due to the slow doubling time of the cell line, VCaP cells were treated with 5-FC and 5-FU for 6 days. This extended treatment increased 5-FU sensitivity in VCaP cells so that 100% cell killing was achieved with 100 µg/ml 5-FU and EC_{50} was 1.8 µg/ml (Figure 49).

However, this result could not be replicated in two further experiments as all cells died including the untreated control wells.

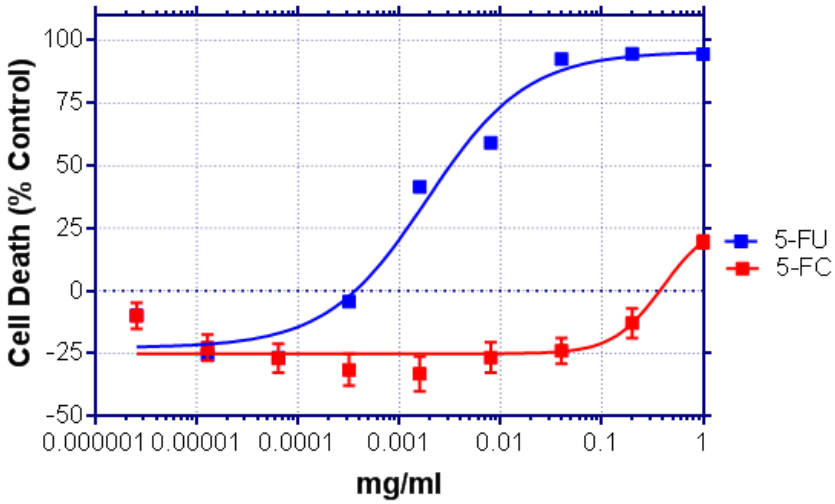


Figure 49 Extended treatment of VCaP cells with 5-FC and 5-FU for 6 days

VCaP cells were treated as previously described (Figure 47D). MTS was performed after 6 days and cell death calculated relative to untreated control wells. Data shown is mean of duplicates ± SEM from one experiment. 6 day extended treatment increased 5-FU sensitivity in VCaP cells. However this was not replicable in two further experiments.

4.2.3 Combination of Ad5-TV-CU and 5-FC in AR-negative cell lines does not induce 5-FC dependent cytoxicity

4.2.3.1 Dose-dependent cell killing in response to Ad5-TV-CU is generated in the AR-negative cell lines HEK293 and DU145

It was disheartening to see that the results generated in 22RV1 cells with a combination of Ad5-TV-CU and 5-FC could not be replicated in AR-positive LNCaP and VCaP cells. However, it was still important to show that Ad5-TV-CU was inactive in a panel of AR-negative cell lines, including immortalized prostate epithelial cells (PNT1A), primary normal prostate basal epithelial cells (PrEC), metastatic PCa cell lines (PC3, DU145), and human embryonic kidney cells (HEK293) (Figure 50).

To confirm the AR driven specificity of Ad5-TV-CU, HEK293 and DU145 cells were used as AR-negative controls. HEK293 was previously used in luciferase assays, demonstrating the lack of activation of the TMPRSS2 promoter in these AR-negative cells (see section 1.7.1 Table 12). DU145 cells were previously used

in western blot analysis to demonstrate the absence of CD/UPRT expression in these AR-negative cells (Figure 42). Initially, dose-responses to 5-FC and 5-FU were established in both cell lines. Both were sensitive to 5-FU treatment (HEK293 EC_{50} = 1 µg/ml, DU145 EC_{50} =3 µg/ml), but were insensitive to 5-FC up to 100 µg/ml (Figure 50A+C). To further demonstrate the AR, prostate specific expression of CD/UPRT from Ad5-TV-CU, HEK293 and DU145 were infected with increasing doses of Ad5-TV-CU. Infection of HEK293 with the non-replicating Ad5-TV-CU resulted in an EC_{50}-value of 30 ppc. In contrast, DU145 were highly insensitive to infection with Ad5-TV-CU (EC_{50} = 10000 ppc). At the same time DU145 cells were infected with the non-replicating Ad5-GFP resulting in a similar EC_{50}-value, demonstrating the intrinsic toxicity of Ad5-GFP and Ad5-TV-CU in DU145 cells. Due to the high quantities of virus required for studies in DU145 cells, they were no longer used for combination experiments (Figure 50B+D).

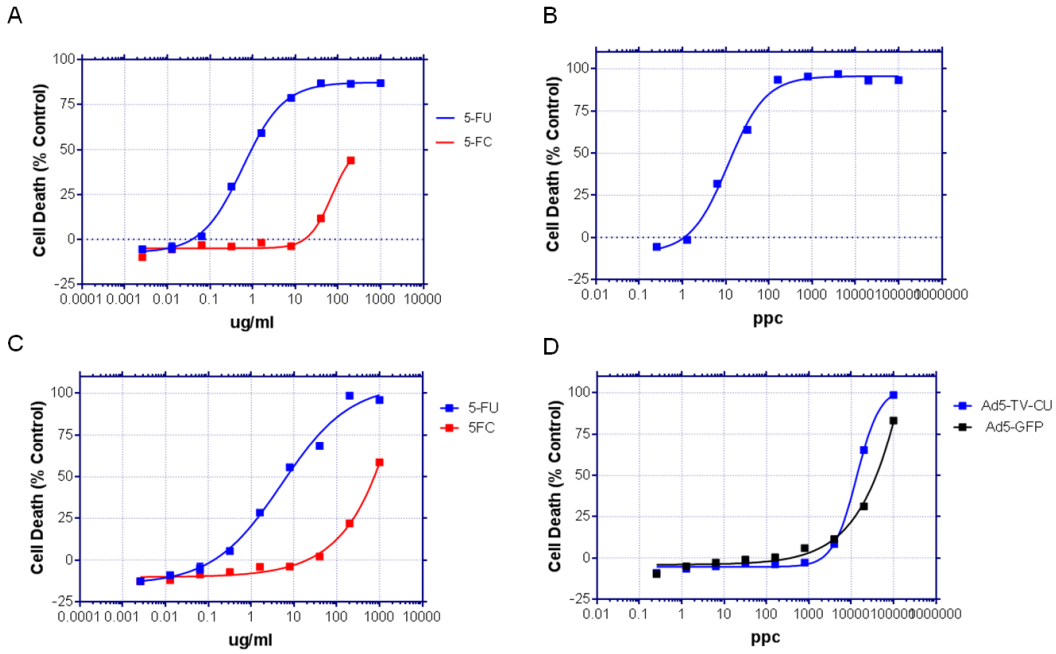


Figure 50 Dose-responses to Ad5-TV-CU were generated in DU145 and HEK293 cells despite no detectable CD/UPRT protein expression

Representative dose-response curves to 5-FC, 5-FU or virus in HEK293 cells (**A+B**) or DU145 cells (**C+D**). **A+C** 5-FC and 5-FU drugs were titrated 1/5 across a 96 well plate from a starting concentration of 1 mg/ml. 10 µl of the titration was added to 90 µl of media containing $1x10^4$ HEK293 or DU145 cells. MTS viability assays were performed after 3 days and cell death calculated relative to untreated control cells. **B+D** Ad5-TV-CU was titrated 1/5 across a 96 well plate and added to 90 µl of media containing HEK293 or DU145 cells. MTS was performed after 3 days and cell viability calculated relative to untreated control wells. Ad5-GFP was used as a control to measure intrinsic toxicity of DU145 cells to adenovirus infection. MTS was performed after 3 days and cell viability calculated relative to untreated control wells. Data shown is mean of duplicates ± SEM from one experiment representative of two.

4.2.3.2 Dose-dependent cell killing by Ad5-TV-CU infection was not observed in primary prostate epithelial cells PrEC or the AR-negative cell lines PC3 and PNT1A.

Due to the difficulties in using HEK293 and DU145 cells as AR-negative control cell lines (discussed previously), three additional prostate cell lines were selected to further confirm the inactivity of Ad5-TV-CU in AR-negative cells, including normal prostate epithelial cells. The AR-negative cell lines PC3 and PNT1A and the primary prostate epithelial cells, PrEC, that are negative for AR at protein level (Figure 41), were infected with Ad5-TV-CU alone and in combination with 50 µg/ml or 100 µg/ml 5-FC. No dose response to Ad5-TV-CU was detected in any of the AR-negative cell lines (Figure 51). This is potentially due to lack of infectability, or more likely lack of AR-dependent CD/UPRT transcription, which is inherently cytotoxic in the AR-positive cell lines, and therefore responsible for Ad5-TV-CU induced cell killing. As could be expected, no increased cytotoxicity was observed in 5-FC prodrug sensitization assays in any of the AR-negative cell lines tested (PC3, PNT1a and PrEC), due to the lack of AR dependent transcriptional activation of Ad5-TV-CU and, as a result the lack of CD/UPRT protein expression.

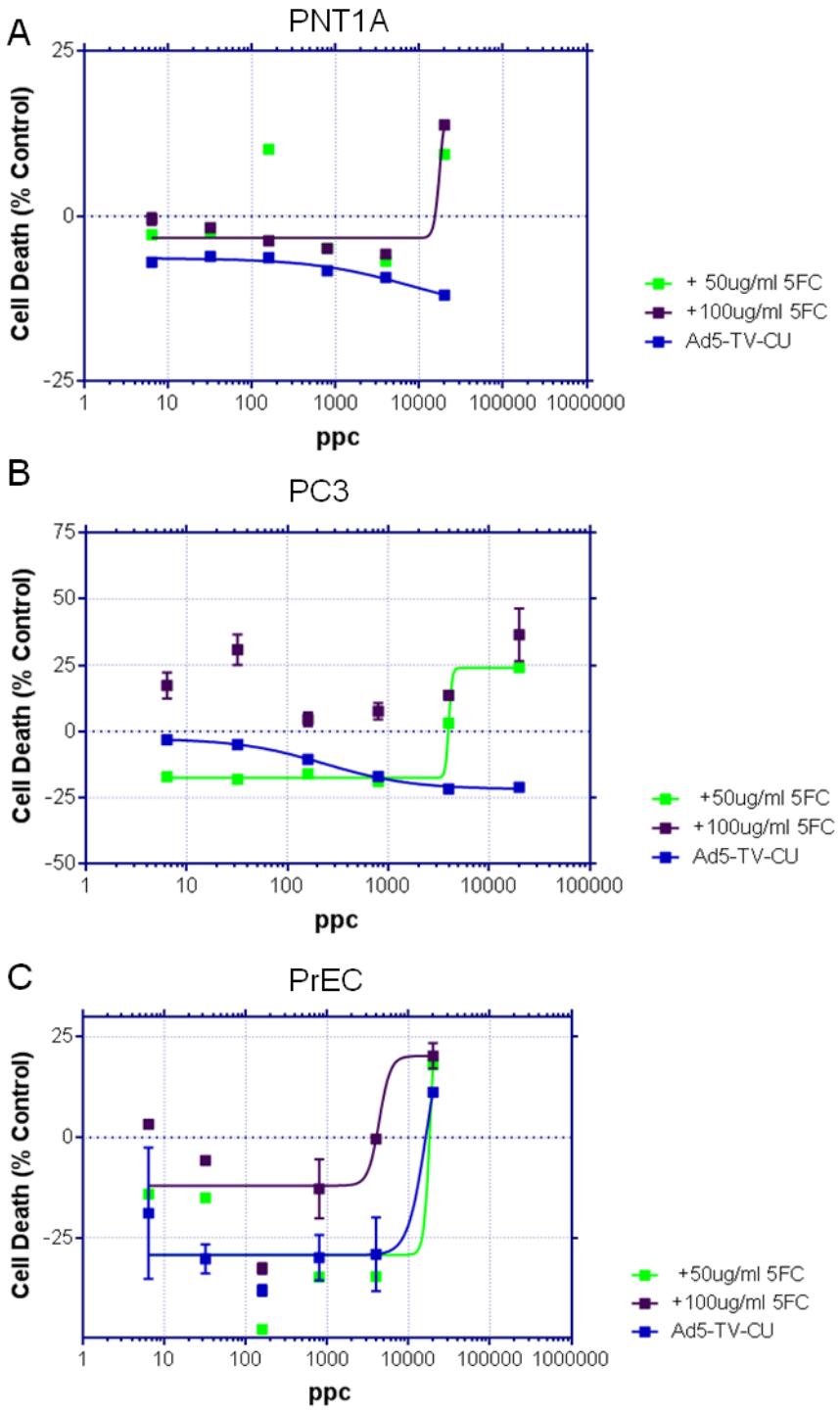
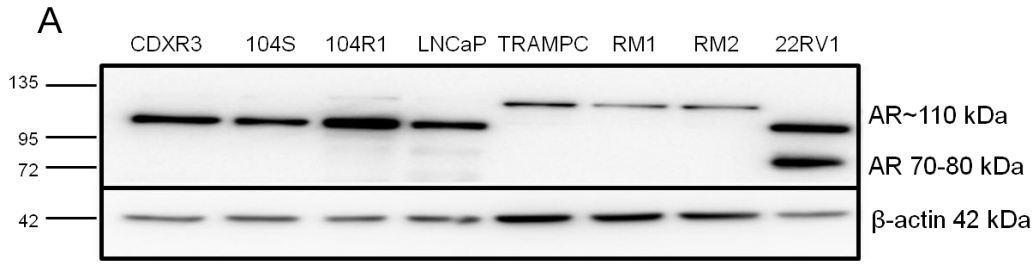


Figure 51 Dose response to Ad5-TV-CU could not be generated by infection of AR-negative cell lines PNT1A and PC3 and the AR-negative primary epithelial cells PrEC

Representative dose response curves to virus in combination with 5-FC prodrug for **A** PNT1A **B** PC3 **C** PrEC. Ad5-TV-CU was titrated 1/5 across a 96 well plate and 10 µl added to 80 µl of media (or 90 µl for virus alone) containing $2x10^4$ PNT1A, PC3 or PrEC cells in combination with 50 µg/ml or 100 µg/ml 5-FC. MTS was performed after 3 days and cell death calculated relative to untreated control wells for virus alone, or drug alone for combinations. No dose response to Ad5-TV-CU was observed. Data shown is mean of duplicates ± SEM from one experiment representative of two.

4.2.4 Identification of new AR expressing cell lines for *in vivo* efficacy studies with Ad5-TV-CU

Due to the poor efficacy of Ad5-TV-CU in combination with 5-FC in VCaP and the failure to detect high levels of CD/UPRT protein post-infection in LNCaP AR-dependent cells, three new LNCaP sublines were tested, LNCaP-104-S, LNCAP-CDXR3 and 104-R1 (see materials and methods section 2.1.1), along with three murine cell lines, RM1, RM2 and TRAMPC. AR expression levels were established in each cell line (Figure 52A). All the LNCaP sublines expressed the same form of androgen receptor, identical to that of the original LNCaP cells (obtained from ATCC) previously used in our studies (110 kDa). AR expression in LNCaP-104R1 and LNCAP-CDXR3 was higher than the parental LNCaP-104-S cells, as previously described (Kokontis et al. 2005). AR was also detected in all murine PCa cell lines, TRAMPC, RM1 and RM2. However, AR in murine cell lines was a different size to AR protein in LNCaP or 22RV1 cells. Protein prediction software suggested that mouse AR is 98 kDa in size, however it appears much larger on the western blot. Further investigation showed that the human AR consists of 919 amino acids while the murine AR is 899 amino acids (20aa smaller) (www.uniprot.org/). Comparison between the two protein sequences showed 88% homology between the two species (Figure 52B). Despite the aa sequence of murine AR being shorter, it has a larger mass than human AR. The difference in weight may therefore be due to post-translational modifications of AR in the murine cell lines, which could potentially affect the binding of AR to the TMPRSS2 promoter elements in the murine cell lines.



B

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Homo            MEVQLGLGRVYPRPPSKTYRGAFQNLFQSVREVIQNPGPRHPEAASAAPPGASLLLLQQQ 60
Mus             MEVQLGLGRVYPRPPSKTYRGAFQNLFQSVREAIQNPGPRHPEAANIAPPGACL------ 54
                ********************************.************. *****.*

Homo            QQQQQQQQQQQQQQQQQETSPRQQQ-QQQGEDGSPQAHRRGPTGYLVLDEEQQPSQPQSA 119
Mus             ------------QQRQETSPRRRRRQQHTEDGSPQAHIRGPTGYLALEEEQQPSQQQAA 101
                            **:******::: **: ******** *******.*:******* *:*

Homo            LECHPERGCVPEPGAAVAASKGLPQQLPAPPDEDDSAAPSTLSLLGPTFPGLSSCSADLK 179
Mus             SEGHPESSCLPEPGAATAPGKGLPQQPPAPPDQDDSAAPSTLSLLGPTFPGLSSCSADIK 161
                 * *** .*:******.*..****** *****:**************************:*

Homo            DILSEASTMQLLQQQQQ---------------EAVSEGSSSGRARERSGAPTSSKDNYLG 224
Mus             DILNEAGTMQLLQQQQQQQQHQQQHQQHQQQQEVISEGSS-ARAREATGAPSSSKDSYLG 220
                ***.**.**********              *.:***** .**** :***:****.***

Homo            GTSTISDNAKELCKAVSVSMGLGVEALEHLSPGEQLRGDCMYAPLLGVPPAVRPTPCAPL 284
Mus             GNSTISDSAKELCKAVSVSMGLGVEALEHLSPGEQLRGDCMYASLLGGPPAVRPTPCAPL 280
                *.*****.************************************.*** ************

Homo            AECKGSLLDDSAGKSTEDTAEYSPFKGGYTKGLEGESLGCSGSAAAGSSGTLELPSTLSL 344
Mus             PECKGLPLDEGPGKSTEETAEYSSFKGGYAKGLEGESLGCSGSSEAGSSGTLEIPSSLSL 340
                .****  **:..*****:*****.*****:*************: ********:**:***

Homo            YKSGALDEAAAYQSRDYYNFPLALAGPPPPPPPPHPHARIKLENPLDYGSAWAAAAAQCR 404
Mus             YKSGALDEAAAYQNRDYYNFPLALSGPPHPPPPTHPHARIKLENPLDYGSAWAAAAAQCR 400
                *************.**********:*** ****.**************************

Homo            YGDLASLHGAGAAGPGSGSPSAAASSSWHTLFTAEEGQLYGPCGGGGGGGGGGGGGGGGG 464
Mus             YGDLGSLHGGSVAGPSTGSPPATTSSSWHTLFTAEEGQLYGP--------------GGGG 446
                ****.****...***.:***.*::******************              ****

Homo            GGGGGGEAGAVAPYGYTRPPQGLAGQESDFTAPDVWYPGGMVSRVPYPSPTCVKSEMGPW 524
Mus             GSSSPSDAGPVAPYGYTRPPQGLTSQESDYSASEVWYPGGVVNRVPYPSPNCVKSEMGPW 506
                *... .:**.*************:.****::*.:******:*.*******.*********

Homo            MDSYSGPYGDMRLETARDHVLPIDYYFPPQKTCLICGDEASGCHYGALTCGSCKVFFKRA 584
Mus             MENYSGPYGDMRLDSTRDHVLPIDYYFPPQKTCLICGDEASGCHYGALTCGSCKVFFKRA 566
                *:.**********:::************************************************

Homo            AEGKQKYLCASRNDCTIDKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGNLKLQEEGEAS 644
Mus             AEGKQKYLCASRNDCTIDKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGNLKLQEEGENS 626
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Homo            STTSPTEETTQKLTVSHIEGYECQPIFLNVLEAIEPGVVCAGHDNNQPDSFAALLSSLNE 704
Mus             NAGSPTEDPSQKMTVSHIEGYECQPIFLNVLEAIEPGVVCAGHDNNQPDSFAALLSSLNE 686
                .: ****:.:**:************************************************

Homo            LGERQLVHVVKWAKALPGFRNLHVDDQMAVIQYSWMGLMVFAMGWRSFTNVNSRMLYFAP 764
Mus             LGERQLVHVVKWAKALPGFRNLHVDDQMAVIQYSWMGLMVFAMGWRSFTNVNSRMLYFAP 746
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Homo            DLVFNEYRMHKSRMYSQCVRMRHLSQEFGWLQITPQEFLCMKALLLFSIIPVDGLKNQKF 824
Mus             DLVFNEYRMHKSRMYSQCVRMRHLSQEFGWLQITPQEFLCMKALLLFSIIPVDGLKNQKF 806
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Homo            FDELRMNYIKELDRIIACKRKNPTSCSRRFYQLTKLLDSVQPIARELHQFTFDLLIKSHM 884
Mus             FDELRMNYIKELDRIIACKRKNPTSCSRRFYQLTKLLDSVQPIARELHQFTFDLLIKSHM 866
                ************************************************************

Homo            VSVDFPEMMAEIISVQVPKILSGKVKPIYFHTQ 917
Mus             VSVDFPEMMAEIISVQVPKILSGKVKPIYFHTQ 899
                *********************************
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Figure 52 AR expression in LNCaP sublines and murine prostate cell lines.

A. Cell lysates were collected and 30 µg run on a protein gel. Rabbit anti-AR antibody was used to reveal AR expression in all cell lines. AR (110 kDa), containing the T877A mutation was detected in all LNCaP sublines, LNCaP-104-S, LNCAP-CDXR3 and LNCaP-104R1 and murine AR was detected in all murine prostate cell lines TRAMPC, RM1 and RM2. Mouse anti-ß-actin antibody was used as a loading control (42 kDa). **B.** Protein sequence alignment between human and murine AR sequences showed 88% homology between the species.

4.2.4.1 The murine cell lines TRAMPC, RM1 and RM2 are highly sensitive to 5-FU but insensitive to infection by AD5-TV-CU.

In order to establish a reliable *in vivo* model to study the activity of Ad5-TV-CU, three AR-positive murine PCa cell lines were investigated for efficacy of Ad5-TV-CU in combination with 5-FC, as murine tumour xenografts could be easily and reliably established in animals with intact immune responses.

TRAMPC, RM1 and RM2 were treated with increasing doses of 5-FC and 5-FU to determine their sensitivity to the non-toxic prodrug 5-FC and the toxic metabolite 5-FU. RM1 and RM2 were highly sensitive to 5-FU, 0.1 µg/ml resulted in 100% cell killing (Figure 53). TRAMPC were more resistant to 5-FU, requiring 4 µg/ml to kill 100% of cells. Whilst all three murine cell lines were sensitive to treatment with 5-FU, much higher doses of the non-toxic 5-FC prodrug were required to achieve 100% cell killing, suggesting that high doses could be used for combination studies.

Dose-response curves to Ad5-TV-CU were generated to determine the inherent toxicity of the CD/UPRT transgene in the murine PCa cells, with 5-FC sensitization assays performed in parallel. The highest concentration of 5-FC that did not induce intrinsic toxicity to the murine cells alone was selected for sensitization assays, as well as a lower concentration of 5-FC. These doses were different for all 3 murine cell lines depending on the sensitivity to 5-FC. MTS assays were performed after 3 days. Only 75% cell killing was observed in RM2 cells infected with Ad5-TV-CU alone at the highest dose ($1x10^5$ ppc), and no increased sensitization was observed with the combination of 5-FC prodrug at either 10 µg/ml or 5 µg/ml. 50% cell killing was observed in TRAMPC cells at the highest dose of 10^5 ppc and the combination with 5-FC enhanced cell killing minimally with 5 µg/ml and 10 µg/ml 5-FC. There was no dose response to Ad5-TV-CU generated in RM1 cells, it was therefore not possible to establish whether the combination with 5-FC enhanced cell killing.

In conclusion, it was not possible to achieve 100% cell killing at the maximum dose of Ad5-TV-CU ($1x10^5$ ppc) in any of the murine PCa cell lines, and was therefore impossible to establish accurate EC_{50} values. Due to the difficulties in establishing a dose response to Ad5-TV-CU, and therefore the inability to perform

prodrug sensitization assays, the murine cell lines were not used for further *in vitro* studies.

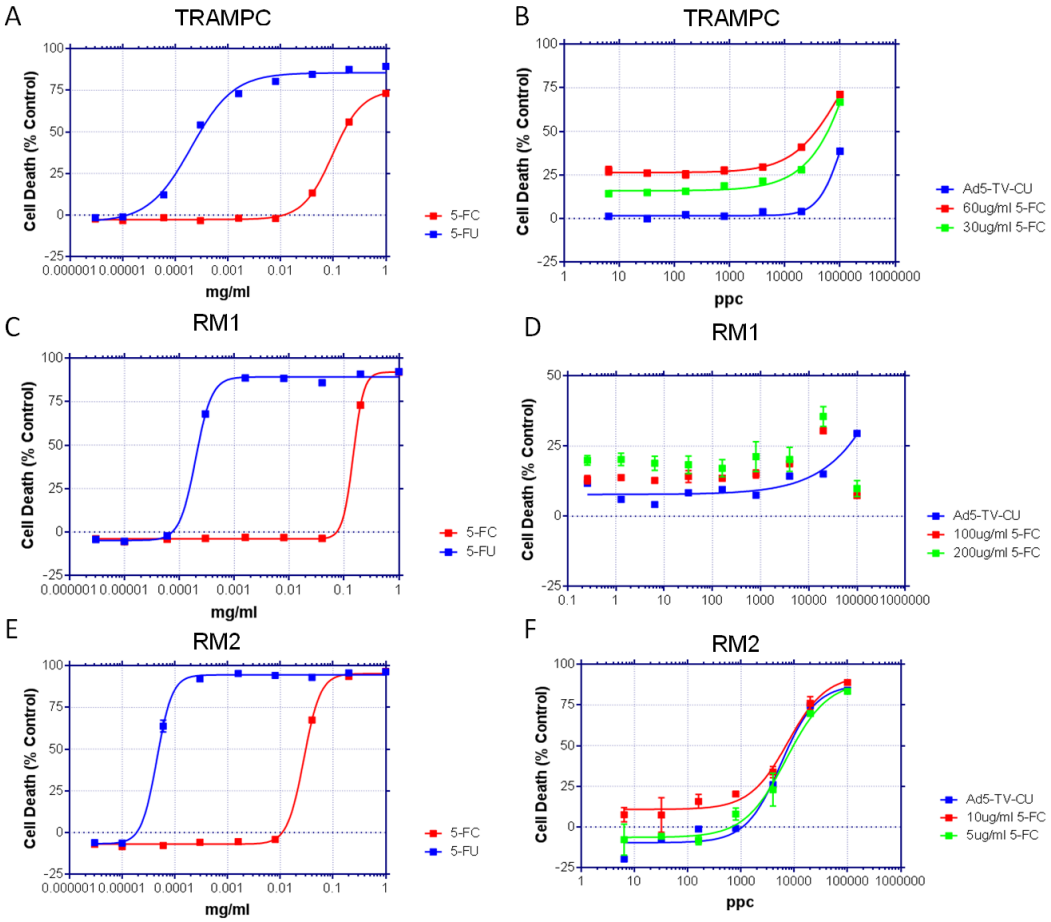


Figure 53 TRAMPC, RM1 and RM2 are sensitive to 5-FU treatment but no increased cell killing is detected with Ad5-TV-CU in combination with 5-FC

Representative 5-FC, 5-FU and virus dose response curves in murine cell lines TRAMPC (**A+B**), RM1 (**C+D**) and RM2 (**E+F**). **A, C** + **E** 5-FC and 5-FU drugs were titrated 1/5 across a 96 well plate from a starting concentration of 1 mg/ml. 10 µl of the titration was added to 90 µl of media containing TRAMPC, RM1 or RM2 cells. MTS was performed after 3 days and cell viability calculated relative to untreated control wells. All three murine cell lines were highly sensitive to 5-FU (TRAMPC EC_{50} 0.4 µg/ml, RM1 EC_{50} 0.2 µg/ml and RM2 EC_{50} 3.75 ng/ml). **B, D** + **F** Ad5-TV-CU was titrated 1/5 across a 96 well plate and 10 µl added of the titration added to 90 µl of media containing $2x10^4$ murine PCa cells alone or to 80 µl in combination with a fixed concentration of 5-FC (TRAMPC 30 µg/ml and 60 µg/ml, RM1 100 µg/ml and 200 µg/ml or RM2 5 µg/ml and 10 µg/ml). Cell viability was calculated relative to untreated control wells for virus alone, or drug only control wells for combination. Data shown is mean of duplicates ± SEM from one experiment representative of three.

4.2.4.2 LNCAP-CDXR3, LNCaP-104R1 and LNCaP-104-S cells express CD/UPRT post Ad5-TV-CU infection and are sensitive to 5-FU treatment.

To establish whether the new sublines of LNCaP AR-positive, AR dependent cells would be suitable for studying Ad5-TV-CU *in vivo*, I infected LNCaP-104-S, LNCaP-104R1 and LNCaP-CDXR3 with increasing doses of Ad5-TV-CU at 1000, 2000 and 3000 ppc. I detected dose dependent increases in CD/UPRT transgene expression post infection in all three sublines (Figure 54). CD/UPRT expression was highest in LNCAP-CDXR3 and LNCaP-104R1 cells in comparison to LNCaP-104-S cells, correlating with higher AR expression in these cell lines (Figure 54).

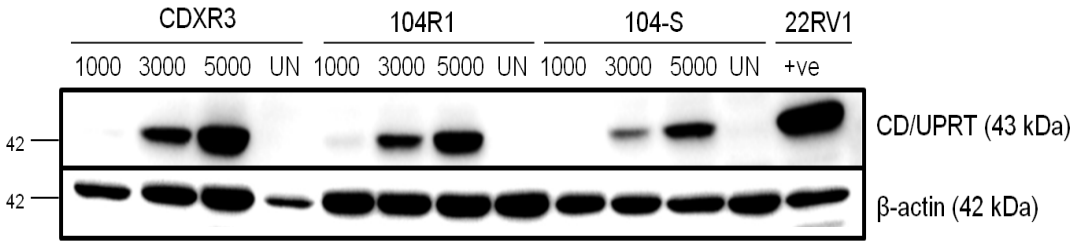


Figure 54 *CD/UPRT* protein expression is detected in LNCAP-CDXR3, LNCaP-104R1 and LNCaP-104-S cells following viral infection

LNCAP-CDXR3, LNCaP-104R1 and LNCaP-104-S cells were infected with 1000 ppc, 3000 ppc and 5000 ppc Ad5-TV-CU and protein lysates collected at 48 h. 30 µg of protein was loaded onto a gel. A dose dependent increase in CD/UPRT expression (43 kDa) was detected in all three cell lines. Sheep Anti-CD antibody was used to reveal CD/UPRT expression. Mouse anti-ß-actin was used as a loading control (42 kDa). A 22RV1 cell lysate previously infected with Ad5-TV-CU was used as a positive control (+ve). Representative blot of three independent experiments.

To establish the toxicity of 5-FC (prodrug) and 5-FU (toxic metabolite) and therefore determine an appropriate concentration at which to add 5-FC in combination with Ad5-TV-CU, dose response curves were generated for both drugs in the LNCaP sublines (Figure 55). 5-FC is slightly toxic in all three LNCaP cell lines at the highest concentration of 1 mg/ml, resulting in ~50% cell death. However, 5-FU is highly toxic to the cells, the average EC_{50} from 3 independent experiments was 1.89±0.88 µg/ml in LNCaP-104-S, 4.11±0.17 µg/ml in LNCAP-CDXR3 and 1.63±0.21 µg/ml in LNCaP-104R1, suggesting that these cell lines will be sensitive to treatment with Ad5-TV-CU in combination with 5-FC if 5-FC is successfully converted to the 5-FU toxic metabolite.

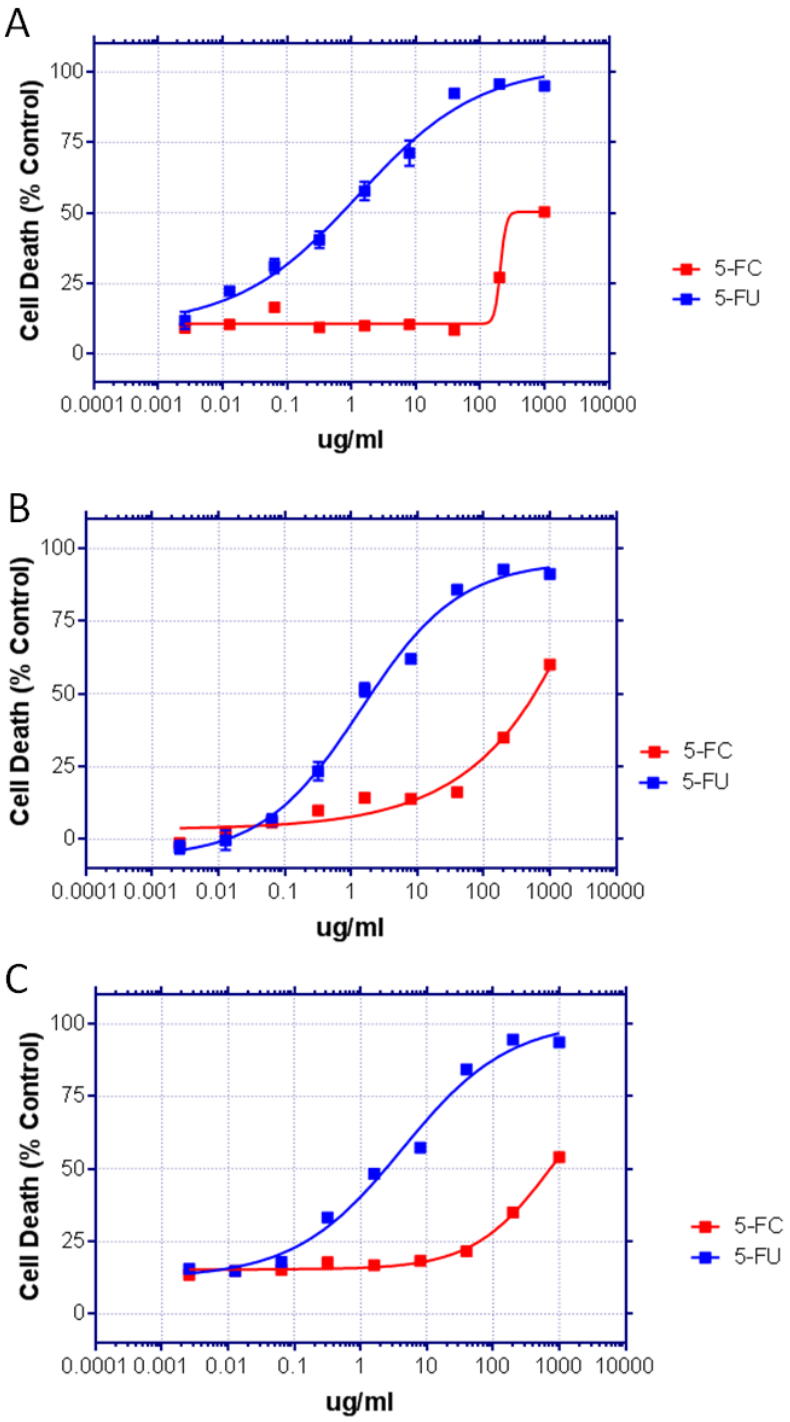


Figure 55 LNCaP-104-S, LNCAP-CDXR3 and LNCaP-104R1 cells are sensitive to treatment with 5-FU, but insensitive to the 5-FC prodrug

5-FC and 5-FU drugs were titrated 1/5 across a 96 well plate from a starting concentration of 1 mg/ml. 10 µl of the titration was added to 90 µl of media containing; **A**. LNCaP-104-S **B**. LNCAP-CDXR3 and **C**. LNCaP-10R1. MTS was performed after 3 days and cell viability calculated relative to untreated control wells. All three cell lines were insensitive to the 5-FC prodrug, but concentrations of 5-FU as low as 4 µg/ml induced ≥ 50% cell death. Data shown is mean of duplicates ± SEM from one experiment representative of three.

4.2.4.3 Combination of Ad5-TV-CU and 5-FC significantly enhances cell killing in LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 cells

In order to establish if the expression of CD/UPRT was sufficient to catalyse the conversion of 5-FC to 5-FU and therefore enhance cell killing in the LNCaP sublines, increasing doses of 5-FC prodrug were added in combination with Ad5-TV-CU in prodrug sensitization assays. Doses of 5-FC prodrug were selected based on EC_{50} values obtained for treatment with 5-FU (ranging from 1-100 µg/ml). Cell killing was enhanced in a 5-FC dose-dependent manner in all three cell lines (Figure 56). EC_{50} in LNCaP-104-S was significantly reduced upon addition of as little as 1 µg/ml 5-FC from 9454± 890 ppc (Ad5-TV-CU alone) to 4052 ± 45.5 ppc (Ad5-TV-CU in combination with 1 µg/ml 5-FC, p-value <0.01, Figure 56B). LNCaP-CDXR3 EC_{50} values were significantly decreased upon addition of 100 µg/ml 5-FC, from 1602 ± 353.6 ppc (Ad5-TV-CU alone) to 478 ± 81 ppc (Ad5-TV-CU in combination with 100 µg/ml 5-FC, p-value <0.01, Figure 56D). LNCaP-104R-1 EC_{50} values decreased upon addition of increasing concentrations of 5-FC (from 5474 ± 1013 ppc (Ad5-TV-CU alone) to 1921 ± 594.3 ppc (Ad5-TV-CU in combination with 100 µg/ml 5-FC, Figure 56F). However due to variability in the biological replicates when generating the data, the decreases in EC_{50} were not significant at any concentration of 5-FC in LNCaP-104R-1. Full details of EC_{50} values ± SEM at increasing concentrations of drug can be found below (Table 29).

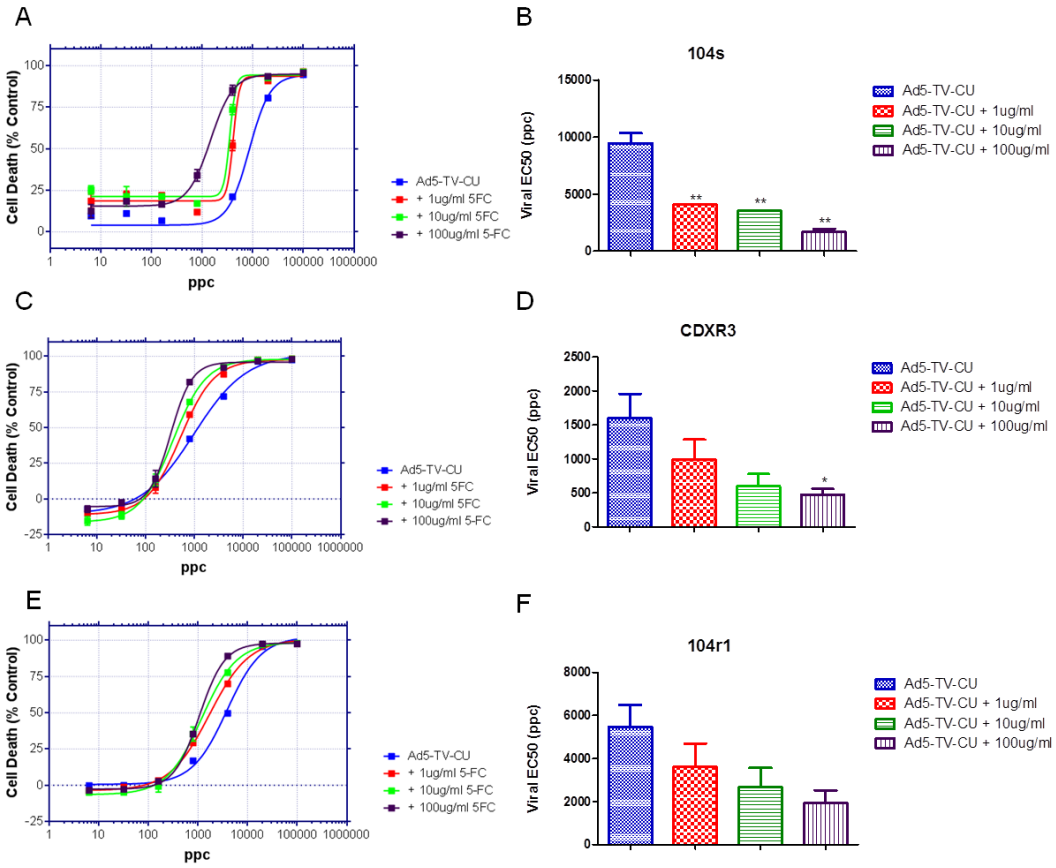


Figure 56 Combination of Ad5-TV-CU and 5-FC in LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 cells generates 5-FC dose dependent shifts in EC_{50} values

A, C & **E** Representative Ad5-TV-CU dose-response curves in combination with 5-FC prodrug. Cells were treated with a 1/5 titration of Ad5-TV-CU starting from $1x10^5$ ppc to 0.2 ppc. 10 µl of the viral dilution was added to 80 µl media containing $2x10^4$ cells/well. 10 µl 5-FC was immediately added in combination with Ad5-TV-CU at fixed doses of 1 µg/ml, 10 µg/ml and 100 µg/ml. MTS assays were performed at three days and cell death calculated relative to untreated control wells, or wells containing drug alone for the combination of Ad5-TV-CU and 5-FC. Data shown is mean of duplicates ± SEM from one experiment representative of four**. B, D** & **F** show histograms of average EC_{50}s for the LNCaP sublines with virus alone, or in combination with 5-FC prodrug. Statistical analysis was performed using the one way ANOVA test with Tukey's multiple comparison test *is p-value <0.05, ** p-value<0.01, (**A + B**) LNCaP-104-S, (**C + D**) LNCaP-CDXR3, (**E + F**) LNCaP-104R1. Data shown is mean of four independent experiments.

Table 29 Average EC_{50} values ± SEM (ppc) for Ad5-TV-CU in combination with increasing doses of 5-FC.

5-FC concentration	LNCAP-CDXR3	LNCaP-104R1	LNCaP-104-S	22RV1
Ad5-TV-CU alone	1602.3 ± 353.6	5474.7 ± 1013.6	9454 ± 890	485.4 ± 96.7
Ad5-TV-CU + 1 µg/ml 5-FC	997.4 ± 286.2	3603 ± 1092.3	4052.5 ± 45.5 **	560.2 ± 118.8
Ad5-TV-CU + 10 µg/ml 5-FC	599.3 ± 178.4	2671.7 ± 888.8	3513.5 ± 30.5 **	327.6 ± 63.1
Ad5-TV-CU + 100 µg/ml 5-FC	478.5 ± 80.9 *	1921.3 ± 594.3	1689 ± 255 **	265.2 ± 52.8

*is p-value <0.05, ** p-value<0.01.

To verify that the increased cell killing upon addition of 5-FC is due to the activation of the 5-FC prodrug by tissue-specific expression of CD/UPRT from Ad5-TV/CU, Ad5-GFP was used as a control virus. Dose response to Ad5-GFP was combined with the same fixed doses of 5-FC at increasing concentrations and MTS cell viability assays performed after 3 days. The cytotoxicity did not change with the addition of 5-FC in combination with Ad5-GFP in any of the LNCaP sublines (Figure 57), confirming that the enhanced cell killing in combination with Ad5-TV-CU was due to CD/UPRT expression from this virus and the subsequent conversion of the non-toxic prodrug (5-FC) to the toxic metabolite (5-FU). EC_{50}-values for Ad5-GFP in comparison to Ad5-TV-CU alone were only similar in LNCaP-104R1 cells (Ad5-TV-CU EC_{50} 5475 ± 1013 ppc, Ad5-GFP EC_{50} 5756 ± 316.7 ppc). EC_{50}-values in LNCaP-CDXR3 cells were much lower when infected with Ad5-TV-CU (EC_{50} 1602.3 ± 353.6 ppc) in comparison to Ad5-GFP (EC_{50} 3707 ± 1174 ppc). The higher levels of AR expression in LNCaP-CDXR3 resulted in higher CD/UPRT expression, which is intrinsically toxic to the cells. Ad5-GFP induced more cell killing (EC_{50} 2245 ± 423 ppc) than Ad5-TV-CU (EC_{50} 9454 ± 890 ppc) in LNCaP-104-S cells, which is most likely due to the lower levels of AR dependent transcriptional activation of Ad5-TV-CU and as a result lower levels of CD/UPRT expression.

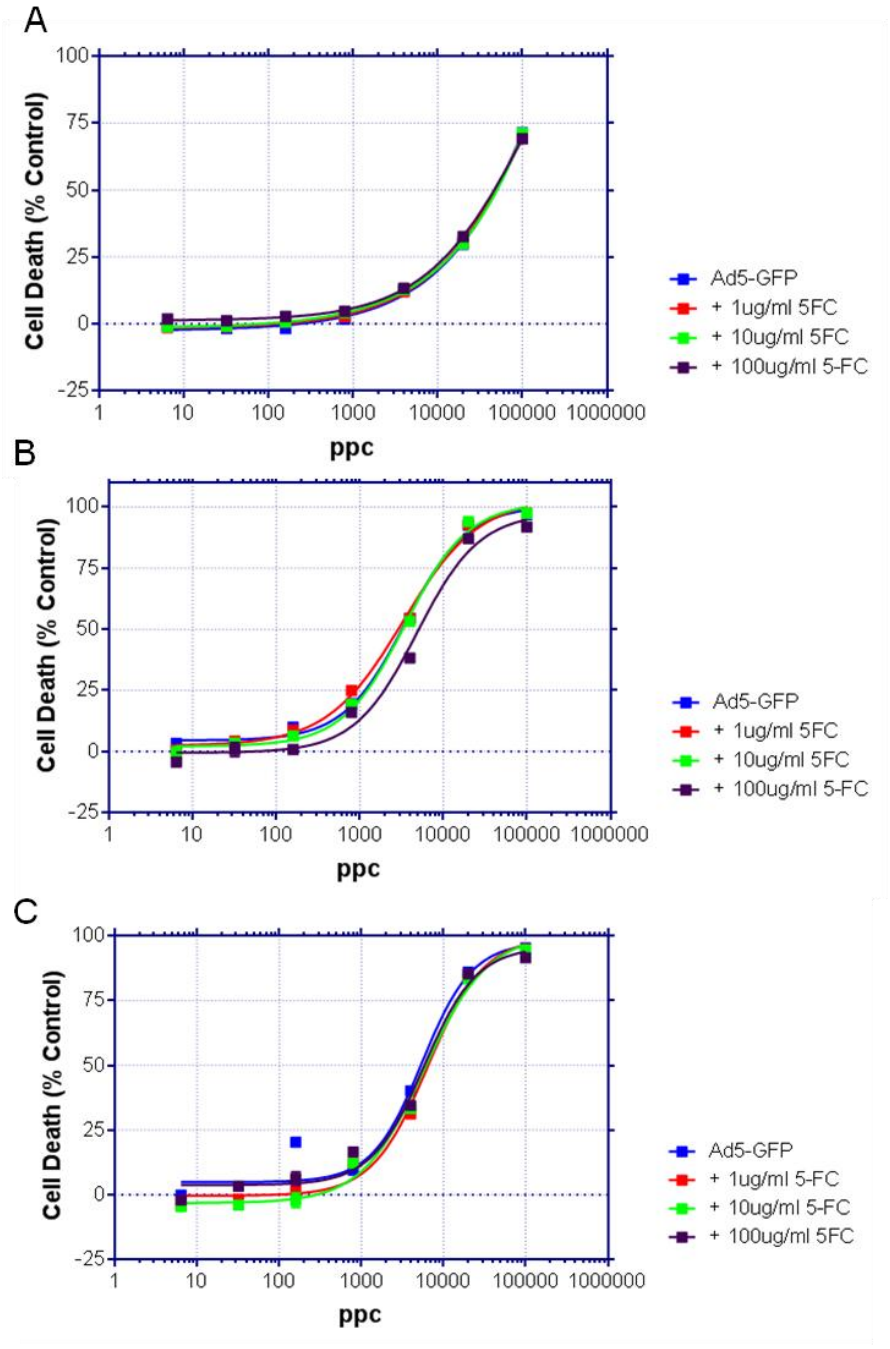


Figure 57 Combination of Ad5-GFP and 5-FC in LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 cells did not generate dose dependent shifts in dose response

Ad5-GFP was titrated 1/5 from 10^5 ppc to 0.2 ppc. 10 µl of the viral dilution was added to 80 µl media containing $2x10^4$ cells/well (or 90 µl for virus alone). 10 µl of 5-FC was immediately added in combination with Ad5-TV-CU at fixed doses of 1 µg/ml, 10 µg/ml and 100 µg/ml. MTS assays were performed at three days and cell death calculated relative to untreated control wells, or wells containing drug alone for the combination of Ad5-TV-CU and 5-FC. No 5-FC dose dependent increased cytotoxicity was observed in any of the three cell lines tested. **A** LNCaP-104-S **B** LNCaP-CDXR3 and **C** LNCaP-104R1. Data shown is mean of duplicates ± SEM from one experiment representative of three.

To demonstrate that enhanced cell killing in LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 was due to target cell sensitization with the combination of Ad5-TV-CU and 5-FC prodrug, doses of Ad5-TV-CU that would kill 10, 20 or 30% (EC_{10}, EC_{20} or EC_{30}) of the cells alone were selected and added in combination with a sub-optimum dose of 5-FC, which did not induce cell death alone (10 mg/ml), but had demonstrated proven increased cytotoxicity in combination with Ad5-TV-CU in the LNCaP sublines. Addition of 5-FC alone at a fixed concentration of 10 µg/ml caused slight toxicity in LNCaP-104-S and LNCaP-CDXR3 cells (<3%) and no toxicity in LNCaP-104R1 cells. As expected, after the addition of 5-FC, cell killing was enhanced in all three cell lines compared with equal doses of Ad5-GFP control virus in combination with 10 µg/ml 5-FC (Figure 58). Due to the variable nature of the cell viability assay the percentage of cell death induced by Ad5-TV-CU varied between experiments, however the extent of cell death induced by the combination of Ad5-TV-CU and 5-FC was consistently higher than the theoretical additive value of Ad5-TV-CU in combination with 5-FC in all five experimental repeats performed in all three cell lines. A significant increase in cell killing was observed in LNCaP-104R1 cells with the combination of Ad5-TV-CU at EC_{20} and 10 µg/ml 5-FC (p-value <0.05), which became highly significant when Ad5-TV-CU was added at EC_{30} in combination with 10 µg/ml 5-FC (p-value <0.01). There were no significant increases in cell killing when Ad5-GFP was added in combination with 5-FC at any dose.

These results demonstrate that Ad5-TV-CU was able to sensitize the LNCaP sublines to 5-FC in a dose-dependent manner. Failure to replicate the sensitization using Ad5-GFP in combination with 5-FC demonstrated that the increased sensitization was specific to cells treated with Ad5-TV-CU and likely the conversion of 5-FC to toxic 5-FU. Ad5-GFP was therefore selected as a control virus for future *in vivo* studies in LNCaP-104-S and LNCaP-CDXR3 tumour xenograft models.

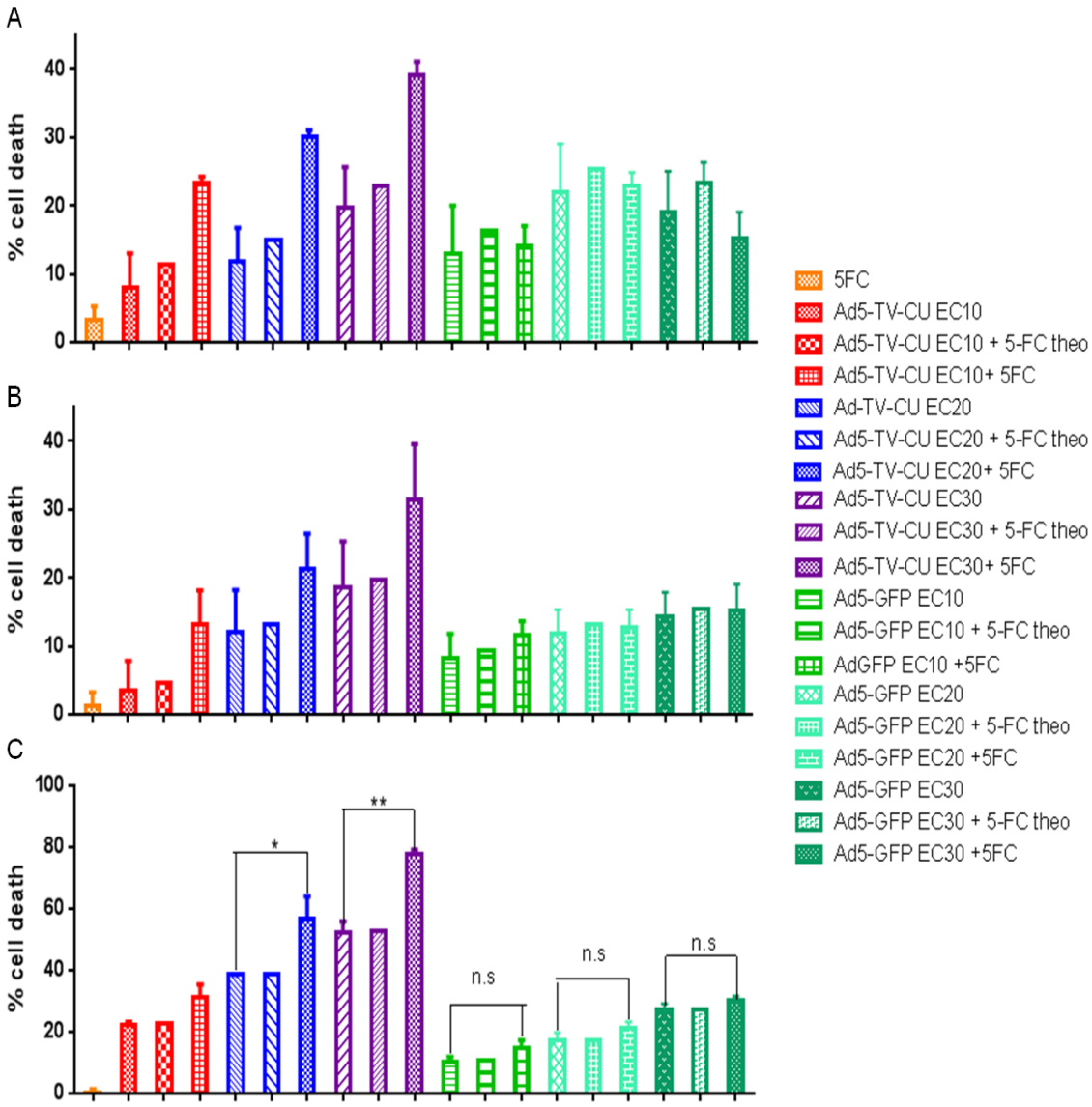


Figure 58 Cell killing is enhanced upon addition of the 5-FC prodrug in LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 cells.

Fixed doses of Ad5-TV-CU were selected that would kill 10%, 20% or 30% of the LNCaP sublines (EC_{10}, EC_{20} or EC_{30}). The same ppc count was fixed for Ad5-GFP. Ad5-TV-CU was added in combination with a fixed dose of 5-FC (10 µg/ml) that had previously been shown to increase cytotoxicity in combination with Ad5-TV-CU. Cell death was determined 3 days post treatment by MTS and cell viability calculated relative to untreated control wells. Histograms show relative cell death as a percentage of total living cells. **A** LNCaP-104-S, **B** LNCaP-CDXR3 and **C** LNCaP-104R1 Results are a mean of triplicate wells from five independent experiments ± SEM. Statistical analysis was performed using the one way ANOVA test with Tukey's multiple comparison test. *is p-value <0.05, ** p-value<0.01, n=3. theo= theoretical additive value.

4.2.4.4 STR profiling highlights genetic differences between the parental LNCaP-104-S cells and LNCaP-CDXR3 and LNCaP-104R1 sublines.

In order to confirm the parentage of the LNCaP Chicago sublines (Dr Kokontis), STR profiling was performed on genomic DNA extracted from LNCaP (ATCC), LNCaP104-S, LNCaP-CDXR3 and LNCaP-104R1 (the genome centre, Barts Cancer Institute). All eight core loci of our initial LNCaP cells matched the ATCC reference. However, STR profiling revealed differences between the LNCaP ATCC reference profile and LNCaP-104-S cells in 3/8 core markers. Additionally, LNCaP-CDXR3 differed by 7/8 core markers and LNCaP-104R1 differed by 6/8 core markers in comparison to the ATCC LNCaP reference respectively. LNCaP-CDXR3 differed by 5/8 core markers and LNCaP 104-R1 differed by 6/8 core markers in comparison to the parental LNCaP-104-S respectively (Table 30).

Table 30 STR profiling of LNCaP sublines

Powerplex16 Loci	**ATCC reference** CRL-1740	**Customer sample** LNCaP ATCC	LNCaP-104-S	LNCaP-CDXR3	LNCaP-104R1
AMELO	X,Y	X,Y	X,X	X,X	X,X
THO1	9,9	9,9	9,9	9,10	9,11
D5	11,12	11,12	11,12	9,11,12	9,?,11,12
D13	10,12	10,12	10,12	10,12	10,12
D7	9.1,10.3	9.1,10.3	9,?,11	?,?,11	?,?,?
D16	11,11	11,11	11,12	10,11,12	10,11,12
CSF	10,11	10,11	10,11	10,12,?	10,12,?
VWA	16,18	16,18	15,17,18	17,18,19,20	17,18,20
TPOX	8,9	8,9	8,9	8,8	8,9

Table shows comparison of core marker. STR core markers that differed are highlighted in red.

4.2.4.5 CD/UPRT protein expression is not stimulated by Mibolerone or inhibited by Bicalutamide after Ad5-TV-CU infection in LNCaP 104-S cells.

In previous studies I was unable to detect an increase/decrease in CD/UPRT protein expression after treatment with 1 nM mibolerone or 5 µM Bicalutamide in

combination with Ad5-TV-CU in the androgen-independent cell line 22RV1. This was thought to be due to the mutated androgen receptor in this cell line, that is constitutively active, and therefore unaffected by stimulation or inhibition. To test the AR responsiveness of Ad5-TV-CU in an androgen-dependent cell line, LNCaP-104-S cells were infected with Ad5-TV-CU and stimulated with 1 nM mibolerone. This experiment was repeated three times and reproduced the original findings, with no significant differences in transgene expression after Mibolerone- treatment. A representative blot is shown in (Figure 59). LNCaP-104-S cells were also infected with Ad5-TV-CU, treated with 5 µM Bicalutamide and CD/UPRT protein expression assessed by western blot. As I observed with mibolerone treatment, there was no significant effect on transgene expression after treatment with Bicalutamide. However, visualisation by western blot is not the most sensitive method to measure small changes in transgene expression, a more sensitive approach would be by Q-PCR or luciferase reporter assay that are able to detect small changes in transgene expression levels (discussed later, section 6.2.3).

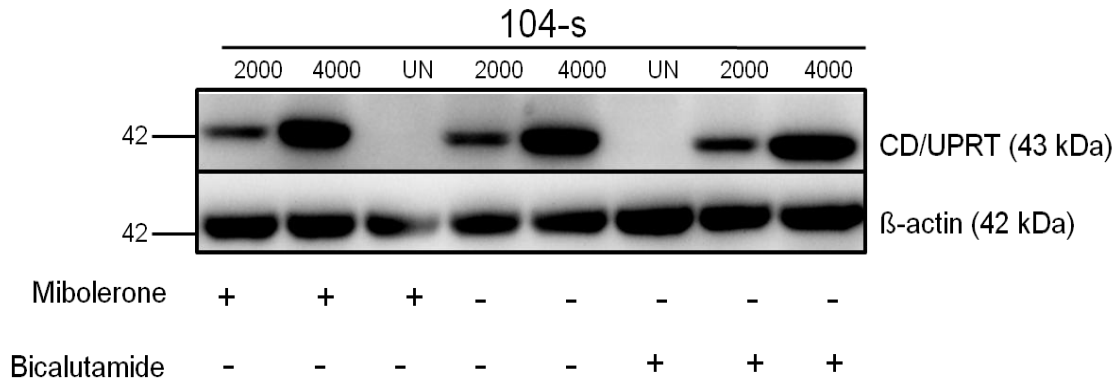


Figure 59 CD/UPRT expression following treatment with 1 nM mibolerone and 5 µM Bicalutamide

LNCaP-104-S cells were infected with 2000 ppc and 4000 ppc Ad5-TV-CU in 10% C/S serum. Two hours after infection the cells were treated with mibolerone (1 nM) or Bicalutamide (5 µM). Cells were lysed after 48 h and 30 µg of protein run on a gel. There was no significant increase or decrease in the CD/UPRT protein expression after stimulation with mibolerone or bicalutamide respectively. Sheep Anti-CD antibody was used to reveal CD/UPRT expression (43 kDa). Mouse Anti-ß-actin was used as a loading control (42 kDa). Representative blot of three independent experiments.

4.2.5 AR-positive BCa and the role of ER in TMPRSS2 promoter activation

4.2.5.1 Combination of Ad5-TV-CU and 5-FC does not enhance cell killing in the AR-positive BCa cell lines, MCF7 and MM453.

Having previously demonstrated the activity and efficacy of Ad5-TV-CU in AR-positive 22RV1 cells and the LNCaP sublines LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 it was desirable to establish if Ad5-TV-CU was also active in MCF7 and MM453 BCa cell lines, that have been previously reported to express AR (Cochrane et al. 2014), and could potentially be used as a BCa therapy alongside PCa therapy. Despite publications that show AR expression in MCF7 cells, AR was undetectable under the conditions used in my study (Figure 41). Nevertheless, MCF7 and MM453 cells were tested for sensitivity to 5-FC/5-FU with and without simultaneous infection with Ad5-TV-CU (Figure 60). MCF7 cells were more sensitive to 5-FU (EC_{50} 3 µg/ml) than MM453 (EC_{50} 50 µg/ml) (Figure 60 A+C). Both cell lines were resistant to treatment with 5-FC up to doses of 10 µg/ml. When Ad5-TV-CU was combined with 5-FC in MCF7 cells there was no dose response to virus alone, or in combination with 5-FC (Figure 60B). This is most likely due to low levels of AR expression in this cell line and limited CD/UPRT expression as a result (Cochrane et al. 2014). A dose response to Ad5-TV-CU was generated in MM453 cells, which were previously reported to express higher levels of AR, meaning higher levels of CD/UPRT expression post-infection (Cochrane et al. 2014). However, there was no shift in the dose-response to Ad5-TV-CU in combination with 5-FC (Figure 60D). This may be due to the poor cell killing effect of 5-FU in this cell line, as much higher doses of 5-FU are required to induce cell killing, or more likely poor expression of CD/UPRT post infection in this cell line. CD/UPRT expression was never quantified due to no efficacy with Ad5-TV-CU alone or in combination with 5-FC. Further investigation in future studies with more AR-positive BCa cell lines could be interesting in the future, in order to increase the clinical applications for Ad5-TV-CU as a potential therapy for estrogen receptor negative (ER-), AR+ BCa.

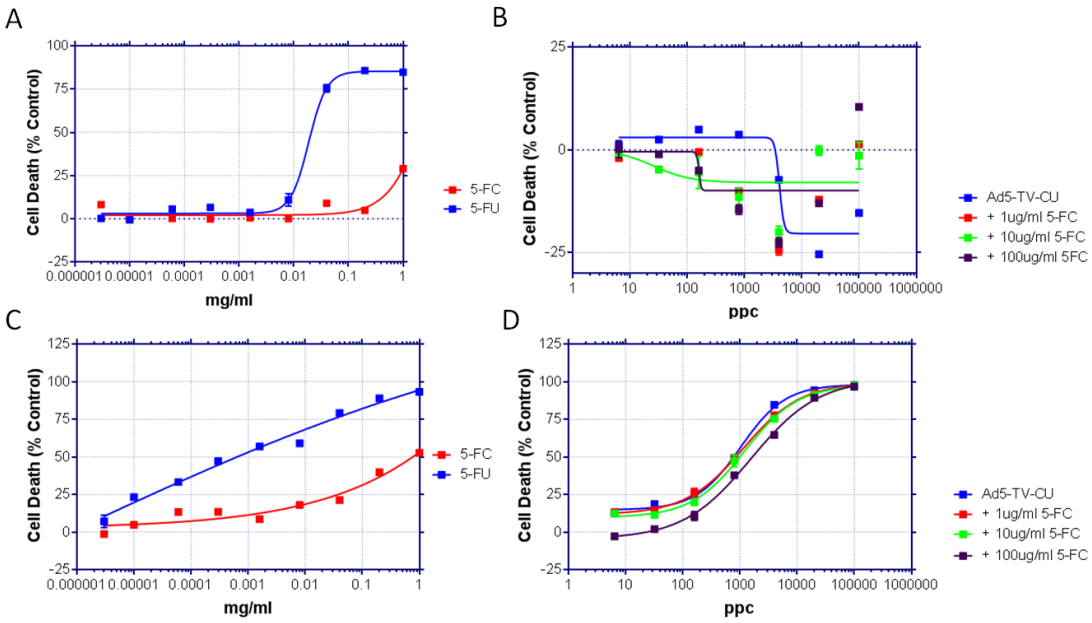


Figure 60 Dose response to Ad5-TV-CU could only be generated in MM453 AR-positive breast cell line following Ad5-TV-CU infection

Representative virus and drug dose response curves in MCF7 (**A + B**) and MM453 (**C + D**) cells for 5-FC, 5-FU (**A + C**) and Ad5-TV-CU in combination with increasing doses of 5-FC (**B + D**). **A + C** 5-FC and 5-FU drugs were titrated 1/5 across a 96 well plate from a starting concentration of 1 mg/ml. 10 µl of the titration was added to 90 µl of media containing $1x10^4$ MCF7 or MM453 cells. MTS assays were performed at 3 days and cell death calculated relative to untreated control wells**. B + D** Ad5-TV-CU was titrated 1/5 across a 96 well plate and added to 80 µl of media containing MCF7 or MM453 cells in combination with 1 µg/ml, 10 µg/ml and 100 µg/ml or 90 µl media for virus alone. MTS assays were performed at 3 days and cell death calculated relative to untreated control wells, or wells containing drug alone for the combination of Ad5-TV-CU and 5-FC. Data shown is mean of duplicates ± SEM from one experiment representative of three.

4.2.5.2 Investigating the activation of the TMPRSS2 promoter by ER

Previous studies have demonstrated that the *TMPRSS2:ERG* fusion gene can be regulated by estrogen receptor-dependent pathways, and demonstrated increased ERG expression after treatment with the ER-α agonist Raloxifene (Setlur et al. 2008, Bonkhoff and Berges 2009). The sustained expression of the TMPRSS2:ERG fusion transcript in castration-resistant PCas has raised questions as to whether the TMPRSS2 promoter remains active in CRPC through ER-α stimulation. Targeting this novel pathway could provide treatments for late-stage CRPC. Therefore, it was important to establish whether the TMPRSS2 promoter in Ad5-TV-CU could be stimulated by ER-α agonists.

In order to establish if ER could activate the TMPRSS2 promoter in Ad5-TV-CU, a panel of prostate cell lysates were analysed on a protein gel and ER-α protein

expression levels established. The ER-α antibody generated a band at 46 kDa (previously shown to correspond to truncated ER-α (Figtree et al. 2003)) that was strong in all PCa cell lines tested and could be a truncated version of ER-α (Figure 61). However, full length ER-α (66 kDa) could only be detected in the MCF7 BCa cell line.

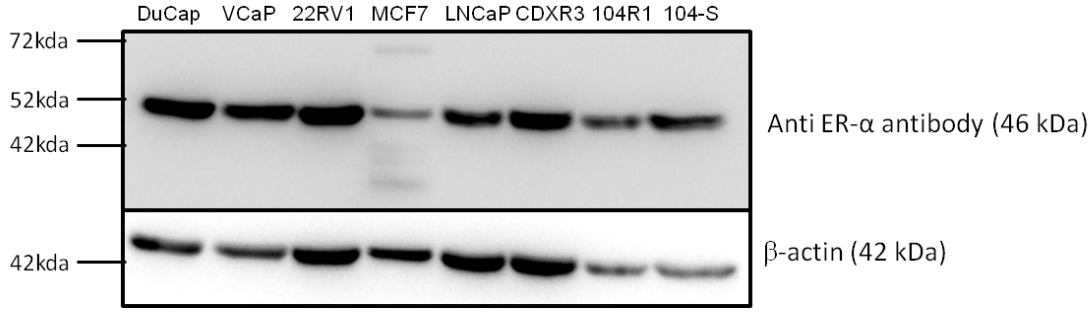


Figure 61 ER-α protein expression in panel of PCa cell lines and MCF7 BCa cell line

Cell lysates were collected and 60 µg run on a protein gel. Rabbit anti-ER-α antibody was used to reveal ER-α expression in all cell lines. Full length ER-α was only detected in the MCF7 BCa cell line. Truncated ER-α was detected in all cell lines. Anti-mouse ß-actin was used as a loading control (42 kDa).

Due to the results generated after infection with Ad5-TV-CU in combination with Mibolerone, and identification of truncated ER-α expression in prostate cell lines, the decision was made to investigate whether estradiol, an ER-α agonist, would increase CD/UPRT transgene expression when added in combination with Ad5-TV-CU in LNCaP-104-S cells.

LNCaP-104-S cells were infected with Ad5-TV-CU in combination with 10 nM estradiol or 1 nM mibolerone. Protein expression was analysed after 48 h by western blot (Figure 62). Treatment with mibolerone did not affect the levels of CD/UPRT expression, as previously seen (section 4.2.4.5). Similarly, no increase in transgene expression was observed upon treatment with estradiol. Due to the relative insensitivity of immunoblotting quantification (described above) I next verified these findings using a reliable and sensitive luciferase reporter assay (section 6.2.3).

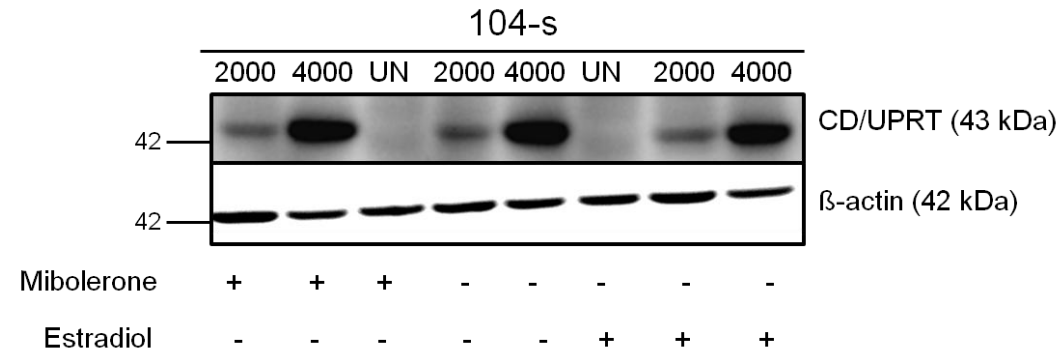


Figure 62 No increase in CD/UPRT expression is detected upon treatment with Mibolerone or Estradiol in combination with Ad5-TV-CU

LNCaP-104-S cells were infected with 2000 ppc and 4000 ppc Ad5-TV-CU in 10% Charcoal Stripped (C/S) serum. Two hours after infection the cells were treated with mibolerone (1 nM) or estradiol (10 nM). A. Cells were lysed after 48h and run on a protein gel. Sheep anti-CD antibody was used to reveal CD/UPRT expression (43 kDa). Mouse anti-ß-actin antibody was used as a loading control (42 kDa). Representative blot of two independent experiments. There was no detectable increase in protein levels of CD/UPRT after stimulation with mibolerone or estradiol.

4.3 DISCUSSION

TMPRSS2 is a prostate specific gene and increased expression in response to AR stimulation has been previously described (Lin et al. 1999). The combination of Ad5-TV-CU and 5-FC significantly enhanced cell killing in AR-positive 22RV1 cells in a 5-FC dose-dependent manner due to AR-dependent transcriptional activation of Ad5-TV-CU. CD/UPRT expression was also detected in a dose-dependent manner after infection of 22RV1 cells with Ad5-TV-CU. However, attempts to demonstrate the AR-responsiveness of Ad5-TV-CU, by upregulation of CD/UPRT expression with the synthetic androgen mibolerone (at 1 nM, chosen as it had previously been shown to stimulate the mmTV-luc vector in luciferase assays (Katrina Sweeny, unpublished studies), or down-regulation with the anti-androgen Bicalutamide did not influence CD/UPRT protein expression levels in 22RV1 cells (see section 4.2.1.3). This could be because the TMPRSS2 promoter is constitutively active in 22RV1 cells, possibly rendering the cells insensitive to AR stimulation or inhibition. These findings are in line with previous studies that have shown that mibolerone treatment of VCaP PCa cells, which harbour wild-type AR protein, strongly upregulated the expression of both PSA and TMPRSS2 androgen responsive genes (Dehm et al. 2008). Whereas, treatment of 22RV1 demonstrated no upregulation, suggesting that AR protein expressed in 22RV1 cells is less responsive to androgen stimulation in comparison to wildtype AR.

Previous findings have demonstrated AR responsiveness of the TMPRSS2 promoter through stimulation with 1 nM of the synthetic androgen R1881 (Lin et al. 1999) and observed the overexpression of ETS family member genes due to fusion of androgen responsive promoter elements in TMPRSS2 to ETS family members (Tomlins et al. 2005). The L promoter present in Ad5-TV-CU contains three putative androgen response elements; it is therefore highly unlikely that this sequence would not respond to transcriptionally active AR.

Very few studies on transcriptional activation of AR in 22RV1 cells have been reported. One previous study, investigating gene expression patterns in 22RV1 cells when stimulated with either 10 nM DHT or 1nM Mibolerone, showed that TMPRSS2 mRNA expression was down-regulated by a factor of 0.6 after 24 h of 10 nM DHT treatment and was also down regulated after 24 h 0.1 nM mibolerone treatment. These findings suggest an inhibitory effect of DHT and mibolerone on

TMPRSS2 expression levels in 22RV1 cells and a more complex pathway of AR transcriptional activation (Hartel et al. 2003). Similarly, previous reports have shown that treatment with 10 µM Bicalutamide alone has a very small inhibitory effect on AR mRNA expression in 22RV1 cells (McCourt et al. 2012) and decreases 22RV1 cell viability by only 10%, whereas similar decreases in cell viability are achieved in LNCaP cells with 1 nM concentrations of Bicalutamide (Seaton et al. 2008).

A further study showed that short isoforms of AR mediated ligand-independent activity in 22RV1 cells (Dehm et al. 2008). Dehm.et.al demonstrated that treatment of VCaP cells, harbouring wildtype AR, with the synthetic androgen mibolerone, upregulated expression of the androgen responsive *PSA* and *TMPRSS2* genes in this cell line, but not in 22RV1 cells (Dehm et al. 2008). Therefore, it is most likely that the constitutively active AR in 22RV1 cells is responsible for the lack of AR agonist/antagonist efficacy. 22RV1 cells express three distinct forms of AR, 1) AR^{EX3dup}, this isoform has a duplicated exon three, consisting of three zinc fingers in its DBD, increasing AR protein size by 5 kDa to 115 kDa, 2) $AR^{1/2/2b}$, a truncated form of AR consisting of the NTD, the first zinc finger in the DBD and 11aa of Exon2b and 3) $AR^{1/2/3/2b}$, a second truncated form of AR consisting of the NTD, the entire zinc finger DBD and an Exon2b derived sequence. The two truncated isoforms $AR^{1/2/2b}$ and $AR^{1/2/3/2b}$ are constitutively active and have been shown to promote an androgen-refractory phenotype (Dehm et al. 2008). These constitutively active forms of AR in 22RV1 cells are responsible for the androgen independency of this cell line, when Dehm.et.al knocked down $AR^{1/2/2b}$ and $AR^{1/2/3/2b}$ specifically, without affecting AR^{EX3dup} expression, androgen-independent 22RV1 cell proliferation was inhibited, as well as androgen-independent expression of AR target genes PSA, TMPRSS2 and NKX3.1 (Dehm et al. 2008). AR^{EX3dup} is therefore the androgen responsive form of AR expressed in 22RV1 cells. However, $AR^{1/2/2b}$ and $AR^{1/2/3/2b}$ are expressed at higher levels than AR^{EX3dup} in 22RV1 cells (Figure 41), consequently overriding androgen responsive AR^{EX3dup} in 22RV1 cells, resulting in decreased sensitivity to AR agonists/antagonists.

These results suggest that Ad5-TV-CU would still be active in androgen-independent cell lines, a common character trait of CRPC. Reactivation of AR pathways through a number of methods leads to the evolution of CRPC (see introduction section 1.3.3). These studies suggest that Ad5-TV-CU could be used in concomitant therapies that target the growth of PCa cells through Bicalutamide,

while simultaneously driving PCa specific cell death through Ad5-TV-CU cytotoxicity, with no effect on efficacy. It may therefore be important to explore the efficacy of Ad5-TV-CU in combination with Bicalutamide in CRPC, with intact AR signalling.

Transgene expression could be detected from VCaP cells after Ad5-TV-CU infection, while in infected LNCaP cells the CD/UPRT levels were very low. It was thought that the possible reasons for this could be: 1) Poor infectability, due to differing expression of coxsackie adenovirus receptor (CAR) and/or integrins on LNCaP PCa cell surface or 2) the activity of the *TMPRSS2* promoter may rely on other transcription factors, which are the limiting factors in activation of Ad5-TV-CU and therefore expression of CD/UPRT.

Infectability was later ruled out as the explanation for poor CD/UPRT transgene expression in LNCaP cells (section 4.2.2.1). This left the absence of transcription factors required for activation of the *TMPRSS2* promoter in Ad5-TV-CU as the probable cause for poor CD/UPRT transgene expression in LNCaP. Previous studies have described the requirement for additional transcription factors that bind together with AR to stimulate transcriptional activation of AR-dependent genes. AR function is activated by several nuclear receptor co-regulators such as SRC-1 and CBP (CREB-binding protein) (Jänne et al. 2000). Absence of any one of these factors could limit the transcriptional activation of AR-dependent genes. Specifically, Menon et al demonstrated that AR requires the chromatin remodelling factor chromodomain helicase DNA-binding protein 8 (CDH8) for the recruitment of AR to the *TMPRSS2* promoter in response to androgen treatment in androgen-dependent LNCaP cells (Menon, Yates, and Bochar 2010). Reduction of CDH8 in this cell line resulted in diminished DHT induced activation of the TMPRSS2 promoter. However, this was not seen in the androgen-independent cell lines 22RV1, PC3 or DU145, suggesting that CDH8 may be important in androgen responsive transcriptional activation. It is therefore highly plausible that the subline of LNCaP use in our group (ATCC) is missing additional transcription factors, for example CDH8 that are essential for AR-dependent activation of the TMPRSS2 promoter in Ad5-TV-CU.

Despite CD/UPRT transgene expression post Ad5-TV-CU infection in VCaP cells and a dose response generated to Ad5-TV-CU, there was no increased cell killing in combination with 5-FC. This appears to be due to resistance of VCaP cells to

the 5-FU toxic metabolite. There are many well know mechanisms of resistance to 5-FU (Zhang et al. 2008), including: 1) The slow doubling time of this cell line, 2) VCaP cells have a mutated p53 status, p53 mutation has previously been show to modulate resistance to 5-FU in colon cancer cell lines (Bunz et al. 1999) and could have a similar effect in VCaP cells. 3) VCaP cells may pump 5-FU out of the cell by efflux systems before 5-FU can have the desired effect on cell killing.

A further study to establish whether the doubling time of VCaP cells was responsible for the resistance to 5-FU did not generate reproducible results. One experiment, in which VCaP cells were subjected to 5-FU treatment for six days, demonstrated increased dose-dependent toxicity to 5-FU in this cell line at day six. However this was not replicable in two further experiments. Due to the difficulties with LNCaP and VCaP AR-positive, AR dependent cell lines and the inconsistent results, these cells were not used for further *in vivo* studies with Ad5-TV-CU.

Contrary to the results generated with the LNCaP cell line in our lab (ATCC), Ad5-TV-CU infection of LNCaP sublines LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104-R1 demonstrated high levels of CD/UPRT protein expression and significantly enhanced 5-FC dose-dependent cell killing in prodrug sensitization assays. In an effort to understand why this was the case, STR profiling was performed on all four LNCaP sublines (genome centre, Barts Cancer Institute). Studies have shown that a minimum of eight core STR markers are required to positively identify human cell lines. Use of these core loci enables a 1 in 10^8 discrimination rate for unrelated cells (ATCC 2014). The STR profile of each LNCaP subline was compared to the ATCC LNCaP reference profile. All eight core STR markers for LNCaP (ATCC) cells in our lab matched the ATCC reference. STR profiling revealed differences between the ATCC reference profile and LNCaP-104-S cells in 3/8 core markers. LNCaP-CDXR3 cell differed by 7/8 core markers and LNCaP-104R1 differed by 6/8 core markers. The differences in the core STR markers in LNCaP-CDXR3 and LNCaP-104R1 cells are most probably due to the castration-resistant phenotype of these cells, resulting in genetic alterations from the parental LNCaP-104-S. Despite these genetic differences all four sublines of LNCaP express high levels of full length AR (110 kDa). It is possible that these genetic differences are responsible for the altered response of LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 to Ad5-TV-CU infection, resulting in CD/UPRT transgene expression and 5-FC dose dependent increased cytotoxicity.

It was therefore desirable to establish whether AR-dependent transactivation of the TMPRSS2 promoter in Ad5-TV-CU could be increased upon AR stimulation with the synthetic AR agonist mibolerone, or decreased with the AR antagonist Bicalutamide in AR-positive, AR dependent LNCaP-104-S cells. As discussed previously, this was not possible in 22RV1 cells, due to the constitutively active forms of AR ($AR^{1/2/2b}$ and $AR^{1/2/3/2b}$) expressed in this cell line, which are responsible for their androgen-independent state (Dehm et al. 2008). Previous studies in LNCaP have shown that addition of the synthetic androgen Mibolerone increases AR transcriptional activity, resulting in upregulation of androgen regulated genes (Young et al. 1991). Conversely, treatment with the anti-androgen Bicalutamide has the opposite effect. However, treatment of LNCaP-104-S cells infected with Ad5-TV-CU in combination with 1 nM mibolerone did not increase CD/UPRT transgene expression, nor was there a decrease in CD/UPRT transgene expression after treatment with 10 µM Bicalutamide. Possible reasons for this could be: 1) lack of additional transcription factors required to activate the TMPRSS2 promoter or 2) lack of interaction between additional AREs that act as enhancers to increase transgene expression from the TMPRSS2 promoter.

Detection of increases or decreases in protein expression after stimulation/inhibition by western blot is an insensitive way to measure AR transcriptional activity. These experiments were later repeated in 22RV1 cells using luciferase reporter assays, that are able to detect subtle increases or decreases in promoter activity (see section 6.2.3).

No cell killing was induced by Ad5-TV-CU in the AR-negative cell lines (PrEC, PNT1A or PC3), except for HEK293 and DU145. Dose response to Ad5-TV-CU in HEK293 cells was due to expression of the E1A gene in HEK293 (Graham et al. 1977), the presence of E1A restores the replication capability of Ad5-TV-CU, resulting in higher cell death due to viral replication and cell lysis. As a result, HEK293 was not used for further studies, as the replicating status of Ad5-TV-CU in this cell line would affect the results of future experiments. A dose response to Ad5-TV-CU was also generated in DU145 cells, however high levels of Ad5-TV-CU were required to induce cell killing in a CD/UPRT independent manner. As expected, no dose response to Ad5-TV-CU was generated in AR-negative metastatic PCa cell line PC3, normal immortalized cell line PNT1A or prostate basal epithelial cell line PrEC.

In conclusion Ad5-TV-CU is not functional in AR-negative cell lines. Additionally, Ad5-TV-CU sensitizes AR-positive 22RV1, LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 to 5-FC prodrug in a dose dependent manner (Table 29), AR-negative cells infected with Ad5-TV-CU remained resistant to the virus and pro-drug at the maximum dose of $1x10^5$ ppc and 500 µg/ml respectively, thereby demonstrating the AR-positive tissue specificity of this construct.

Despite previous publications demonstrating AR expression in MCF7 and MM453 cells (Hall et al. 1994), no enhanced cell killing could be detected with the combination of Ad5-TV-CU and 5-FC. This is most probably due to the insensitivity of MM453 cells to 5-FU, and insufficient production of high levels of 5-FU when Ad5-TV-CU was added in combination with 5-FC. No dose response was generated to Ad5-TV-CU in MCF7 cells, most likely due to the relatively low levels of AR expression in this cell line.

Previous studies have suggested a role for ER-α in the transcriptional activation of TMPRSS2 (Setlur et al. 2008, Bonkhoff and Berges 2009), and as a result upregulation of the TMPRSS2:ERG fusion gene. Due to the difficulties in increasing transgene expression in both 22RV1 and LNCaP-104-S cells with the synthetic AR agonist mibolerone, the potential role of ER-α in TMPRSS2 promoter activation was investigated. All PCa cell lines tested expressed a truncated form of ER-α (46 kDa), but none expressed the full length version (66 kDa). Full length ER-α is weakly expressed in MCF7 breast cells and is responsible for transcriptional activation of ER target genes. The truncated form of ER-α has been found in human endothelial cells and has been shown to activate eNOS in response to estrogen stimulation (Figtree et al. 2003). However, estrogen stimulated transcriptional activation mediated by the 46 kDa truncated form of ER-α is much lower (Figtree et al. 2003) than the 66 kDa full length version.

Similar to the results generated with mibolerone, treatment of LNCaP-104-S cells with Ad5-TV-CU in combination with estradiol, an ER-α agonist did not increase CD/UPRT transgene expression. There are three possible reasons for this: 1) Full length ER-α is not expressed in LNCaP-104-S cells, and cannot therefore bind to response elements in the TMPRSS2 promoter. 2) Examining protein expression by western blot is not sensitive enough to detect increased transgene expression. 3) TMPRSS2 is not activated by ER-α. This experiment was not performed in MCF7 cells due to time limitations, but could be performed for future clarification.

Further studies on more AR+ BCa cell lines and ER+ PCa cell lines would need to be performed to fully elucidate the roles of AR and ER in TMPRSS2 promoter activation and the possible clinical applications of applying Ad5-TV-CU to AR+ BCa.

CHAPTER FIVE

ACTIVITY OF AD5-TV-CU *IN VIVO*

5.1 INTRODUCTION

To confirm that results generated with Ad5-TV-CU in combination with 5-FC *in vitro* could be translated *in vivo*, two xenograft models were established with androgen-independent and dependent LNCaP sublines LNCaP-CDXR3 and LNCaP-104-S respectively. LNCaP-CDXR3 cells express higher levels of AR than the parental LNCaP-104-S cells (Kokontis et al. 2005). These cell lines were chosen as they had previously demonstrated efficacy *in vitro* in combination with 5-FC, and have previously been used by other groups to generate xenografts with reliable results *in vivo* (Kokontis et al. 2005). The remaining cell line that demonstrated efficacy *in vitro*, 22RV1, was not initially chosen for *in vivo* studies, as previous studies in the Halldén lab established that 22RV1 xenograft growth was difficult to control, even with intratumoural injection of replicating virus. Therefore, two pilot studies, each consisting of seven animals, were initiated to confirm efficacy and biodistribution of Ad5-TV-CU in BALB/c male athymic mice bearing LNCaP-104-S and LNCaP-CDXR3 xenografts.

Previous studies in which androgen-dependent LNCaP sublines have been used to induce tumour xenografts suggested the requirement for additional testosterone to allow for tumour growth in nude mice (Resnick and Thompson 2000). Additionally, studies have revealed that BALB/c athymic mice have significantly reduced levels of serum gonadotrophins and testosterone compared with their normal littermates (Rebar et al,1982). Upon receipt of the LNCaP-104-S cells we were recommended by Dr John Kokontis to grow the xenografts *in vivo* with the addition of a testosterone pellet. Consequently, xenografts were grown in male BALB/c athymic mice with a 1.25 mg 60-day slow release testosterone pellet implanted under the skin. LNCaP-104-S cells are an androgen-dependent cell line. However, after further investigation following poor tumour growth, a study by Chuu et al. demonstrated that the response of LNCaP-104-S cells to androgens was biphasic *in vitro.* Furthermore, the showed that addition of a 20 mg testosterone propionate pellet, which raised serum testosterone levels from 2.5 ± 2.0 to 33.4 ± 3.2, had no effect on the growth rate of LNCaP-104-S tumour xenografts *in vivo*. Additionally, growth of these cells is repressed by the androgen receptor antagonist Bicalutamide *in vitro* (Chuu et al. 2006). Further *in vivo* studies were

therefore performed to establish the true effect of additional testosterone on the growth of LNCaP-104-S xenografts.

LNCaP-CDXR3 cells were generated by continued growth of LNCaP-104-S cells for 3 weeks in the presence of 5 μM Casodex (Bicalutamide), an AR antagonist (Kokontis et al. 2005), resulting in the generation of androgen-independent LNCaP-CDXR3 cells. LNCaP-CDXR3 cells feature increased AR expression and transcriptional activity, while cell proliferation is inhibited by androgens both *in vitro* and *in vivo*. As a consequence, LNCaP-CDXR3 tumour xenografts cannot be generated in the presence of androgens and so must therefore be grown in castrated male mice.

Following difficulties establishing LNCaP-104-S xenografts in BALB/c athymic mice, potentially due to evidence of immune cell infiltration, NOD/SCID mice were used for further studies with LNCaP-104-S. These mice are severely immunodeficient, with both T-cell and B-cell development affected in comparison to solely T-cell development in BALB/c athymic mice. Additionally BALB/c athymic mice have intact adrenal, thyroidal and gonadal function, which could interfere with LNCaP-104-S tumour growth.

In the fourth and final pilot study 22RV1 cells were subcutaneously injected into CD-1 athymic male mice. Similar to BALB/c mice, they are T-cell deficient but have normal B-cell function. 22RV1 tumour xenografts have been previously established in the CD-1 mice by the Halldén lab, (unpublished data). They established that $1x10^6$ 22RV1 cells inoculated subcutaneously, resulted in tumour development approximately 2 weeks later, reaching a size of 100 mm^3, suitable for intratumoural injection. In comparison, LNCaP-CDXR3 and LNCaP-104-S, took five to six, and four weeks respectively to develop.

5.2 RESULTS

5.2.1 Pilot study in BALB/C mice with LNCaP-CDXR3 tumour xenografts

5.2.1.1 Ad5-TV-CU in combination with 5-FC impeded growth in two out of three animals.

In the first pilot study, I aimed to investigate efficacy and safety of Ad5-TV-CU in combination with 5-FC *in vivo in* hormonally deficient male BALB/c athymic mice. Seven Castrated male BALB/c athymic mice were inoculated with $1x10^6$ androgen-independent LNCaP-CDXR3 cells in both the left and right flank. Palpable tumours developed in both flanks of three out of seven animals within 3 weeks and three additional animals developed palpable tumours by week 4. One animal failed to develop tumours on either flank and was therefore culled at week 6. Two weeks after palpable tumours developed (between 5 and 6 weeks after the cells were injected) tumours on both flanks in five of the six animals reached >100mm^3 (calculated by W^2 x L x 0.52). A tumour only initially developed on the right flank in the single remaining animal, with a second tumour developing on the left flank by week 10 (Figure 63; purple line), 4 weeks after treatment of the first tumours began, demonstrating the slow growing nature of this cell line.

Once tumours reached >100 mm^3 they were injected intratumourally with $1x10^{10}$vp of either Ad5-TV-CU or Ad5-GFP in either the left or right flank (randomly assigned) on days 1, 3 and 6, for a total of three doses. Animals were subsequently randomised into groups that would receive an intraperitoneal injection of either 5-FC at 100 mg/kg or an equal volume of PBS on days 2, 5, 8 and 15. Tumour volume was monitored twice weekly. Treatment continued until day 15, after which point there was little change in relative tumour volume in any of the groups (except for the control tumours treated with Ad5-TV-CU and PBS) (Figure 63-64). Five of the six animals were culled between days 50 and 60 post treatment initiation. One animal, treated with Ad5-TV-CU and 5-FC, was kept until day 62 post treatment initiation because tumour volume continued to decrease after day 50 (Figure 63).

Only one out of nine control tumours grew >800 mm^3 (Ad5-TV-CU+PBS), suggesting that tumours did not grow well in the BALB/c mice. None of the

tumours treated with Ad5-TV-CU in combination with 5-FC grew larger than 200 mm^3 throughout the entire *in vivo* study. Similarly, only one out of three control tumours treated with Ad5-TV-CU in combination with PBS grew larger than 200 mm^3 and neither of the two tumours treated with Ad5-GFP in combination with PBS grew larger than 90 mm^3 throughout the length of the study. Consequently, tumour regression could not be reliably determined. Therefore, the study was terminated due to the limited and arbitrary results obtained despite the tumours continuing to stay below the Home Office standard guidelines for tumour burden.

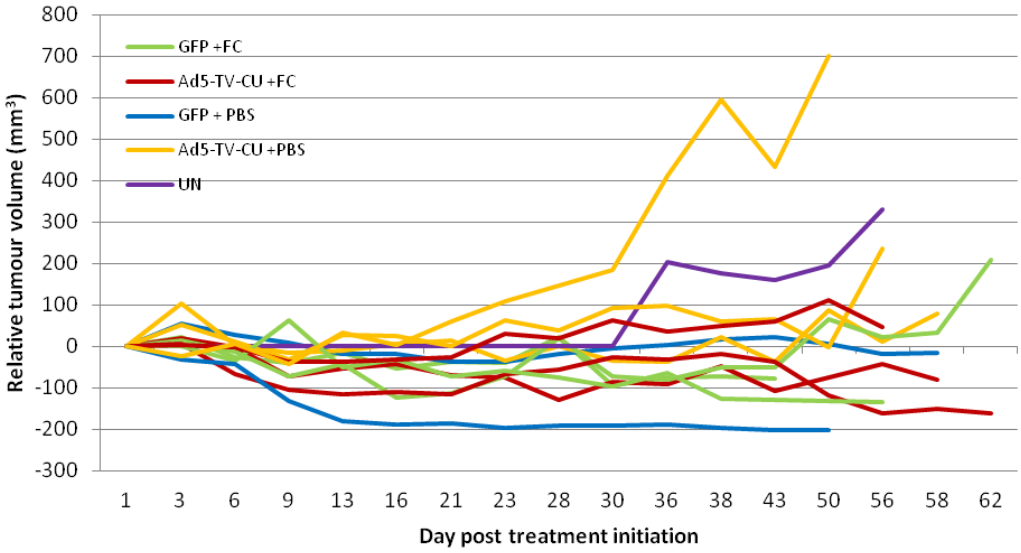


Figure 63 LNCaP-CDXR3 tumours treated with Ad5-TV-CU or Ad5-GFP in combination with 5-FC or PBS generated variable relative tumour growth results

Castrated male BALB/c athymic mice were injected subcutaneously with hormone independent LNCaP-CDXR3 cells. After allowing the tumours to grow for 5 to 6 weeks to 100 mm^3 animals were separated into control groups (Ad5-TV-CU plus PBS, yellow lines, Ad5-GFP plus PBS, blue line and Ad5-GFP plus 5-FC, green lines) or Ad5-TV-CU plus 5-FC (red lines) and the time was designated as day 1. 30 days after treatment initiation a tumour developed in the opposite flank of one animal and was left untreated (purple line). Intratumoural injections of $1x10^{10}$vp of either Ad5-TV-CU or Ad5-GFP were administered on days 1, 3 and 6 and intraperitoneal injections of 100 mg/kg of either 5-FC or PBS given on days 2, 5, 8 and 15. There were two tumours on each animal and each line on the graph is representative of one tumour. Graphs showing matched tumours in each individual animal can be seen in the appendix section 8.2, Figure_Apx1. Tumour growth was normalised relative to the individual starting tumour volume determined on day 1 of treatment to illustrate the effect of treatment on relative tumour growth. n= number of tumours per group. n=3 (Ad5-GFP +5-FC, Ad5-TV-CU+5-FC, Ad5-TV-CU+PBS) n=2 (Ad5-GFP +PBS) n=1 (UN) UN= untreated.

Despite poor tumour growth, treatment with Ad5-TV-CU in combination with 5-FC resulted in a reduction in tumour volume in two out of three animals and in the third animal tumour size did not increase, resulting in inhibition of tumour growth overall (red lines; Figure 63-64). Control animals with LNCaP-CDXR3 xenografts

treated with a combination of Ad5-TV-CU and PBS showed a trend towards increased relative tumour growth in two out of three animals, resulting in increased tumour growth overall (yellow lines; Figure 63-64). The tumour volume did not exceed 200 mm^3 in one animal (Figure 63). These findings suggest that Ad5-TV-CU must be combined with the 5-FC pro-drug to truly inhibit relative tumour growth of LNCaP-CDXR3 xenografts.

Surprisingly, treatment with Ad5-GFP in combination with both 5-FC and PBS also appeared to inhibit the growth of LNCaP-CDXR3 tumour xenografts in four out of five tumours (Figure 63-64). In fact, the biggest decrease in tumour volume was observed in one animal treated with Ad5-GFP in combination with PBS (Figure 63-64). This result was unexpected as Ad5-GFP had been used previously as a control virus *in vitro* in this cell line and found to have no effect on cell growth or viability.

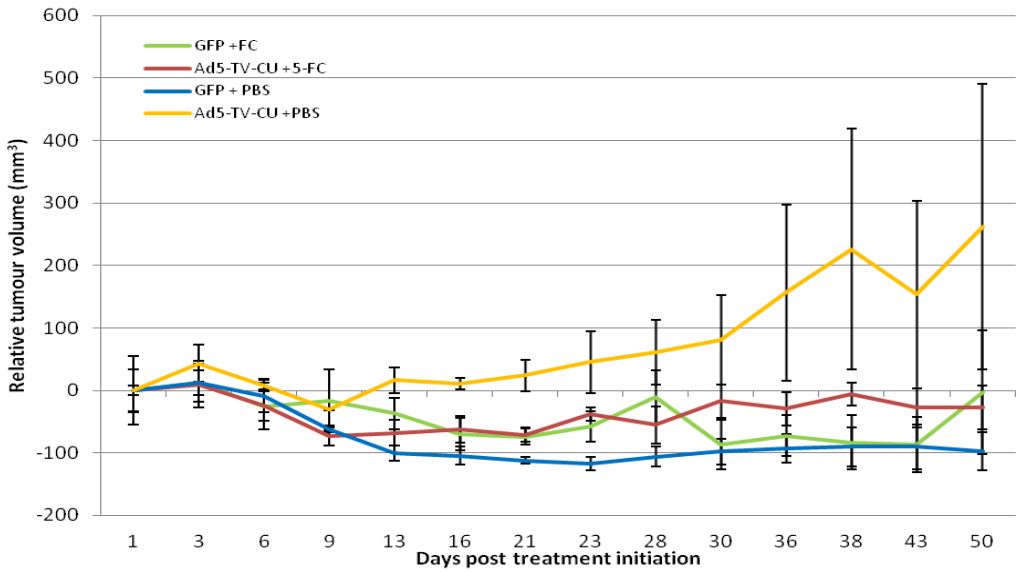


Figure 64 Average relative tumour volume increased with treatment of Ad5-TV-CU in combination with PBS, but decreased with all other treatments

Mice bearing 100 mm^3 hormone independent LNCaP-CDXR3 xenografts were separated into control groups (Ad5-TV-CU plus PBS, yellow line, Ad5-GFP plus PBS, blue line and Ad5-GFP plus 5-FC, green line) or Ad5-TV-CU plus 5-FC (red line) and the time was designated as day 1 for the first treatment. Data shows the average tumour volume measurements in mm^3 ±SEM over a total period of 57 days. Tumour volume was normalised relative to the individual starting tumour volume in each animal determined on day 1 of treatment and averaged, to illustrate the effect of treatment on relative tumour growth and average relative tumour growth plotted over 50 days ± SEM. n= number of tumours per group. n=3 (Ad5-GFP +5-FC, Ad5-TV-CU+5-FC, Ad5-TV-CU+PBS) n=2 (Ad5-GFP +PBS) n=1 (UN) UN= untreated. p>0.05 by one-way anova.

5.2.1.2 Treatment with Ad5-TV-CU in combination with 5-FC resulted in tumour regression in one animal with LNCaP-CDXR3 xenografts.

One animal bearing LNCaP-CDXR3 tumour xenografts on the left and right flank was injected with Ad5-TV-CU in the left flank and Ad5-GFP in the right flank on days 1, 3 and 6. An intraperitoneal injection of 100 mg/kg 5-FC was given on days 2, 5, 8 and 15. Initially, tumour volume in both flanks decreased from the start of treatment (day 1) up to the end of treatment (day 15) and steadily began to rise after treatment ended up to ~160 mm^3 for Ad5-GFP in combination with 5-FC and 130 mm^3 for Ad5-TV-CU in combination with 5-FC at day 23 (Figure 65A). However, post day 37 of treatment initiation the LNCaP-CDXR3 tumour xenograft treated with Ad5-TV-CU steadily decreased in volume from 180 mm^3 to 36.8 mm^3, whilst the xenograft treated with Ad5-GFP continued to grow up to 440 mm^3 until day 62, at which point the animal was culled (Figure 65A). The LNCaP-CDXR3 tumour xenograft treated with Ad5-TV-CU had almost completely regressed such that only a small flat tumour could be dissected, whilst the tumour treated with Ad5-GFP had continued to grow and was large and well rounded (Figure 65B).

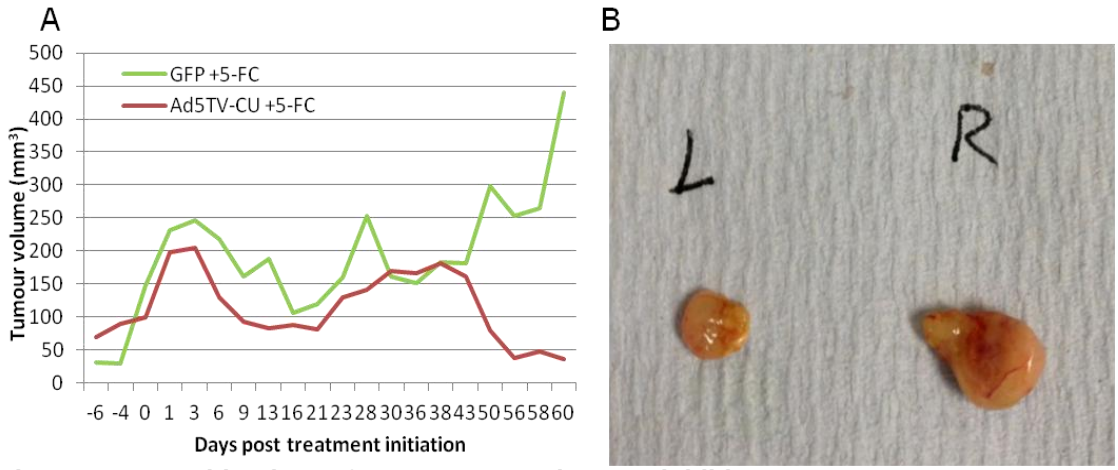


Figure 65 Combination of A5-TV-CU and 5-FC inhibits LNCaP-CDXR3 tumour xenograft growth

A. Tumour volume measurements from one animal treated with either Ad5-GFP (green line) or Ad5-TV-CU (red line) in combination with 5-FC. **B**. LNCaP-CDXR3 tumour xenografts from one animal injected intratumourally with Ad5-TV-CU (L) or Ad5-GFP (R) in combination with 5-FC.

5.2.1.3 Immune cell infiltration of LNCaP-CDXR3 tumours

In order to better understand the poor relative tumour growth of LNCaP-CDXR3 xenografts, H&E staining was performed on treated LNCaP-CDXR3 xenograft tissue. There was no difference in the morphology of xenografts infected with Ad5-GFP in comparison to those infected with Ad5-TV-CU in combination with either 5-FC or PBS (Figure 66A-H). On closer inspection there was no evidence of an inflammatory response in xenografts injected with both Ad5-GFP and Ad5-TV-CU (Figure 66B, D, F and G). However there was evidence of necrosis in xenografts treated with Ad5-TV-CU in combination with 5-FC.

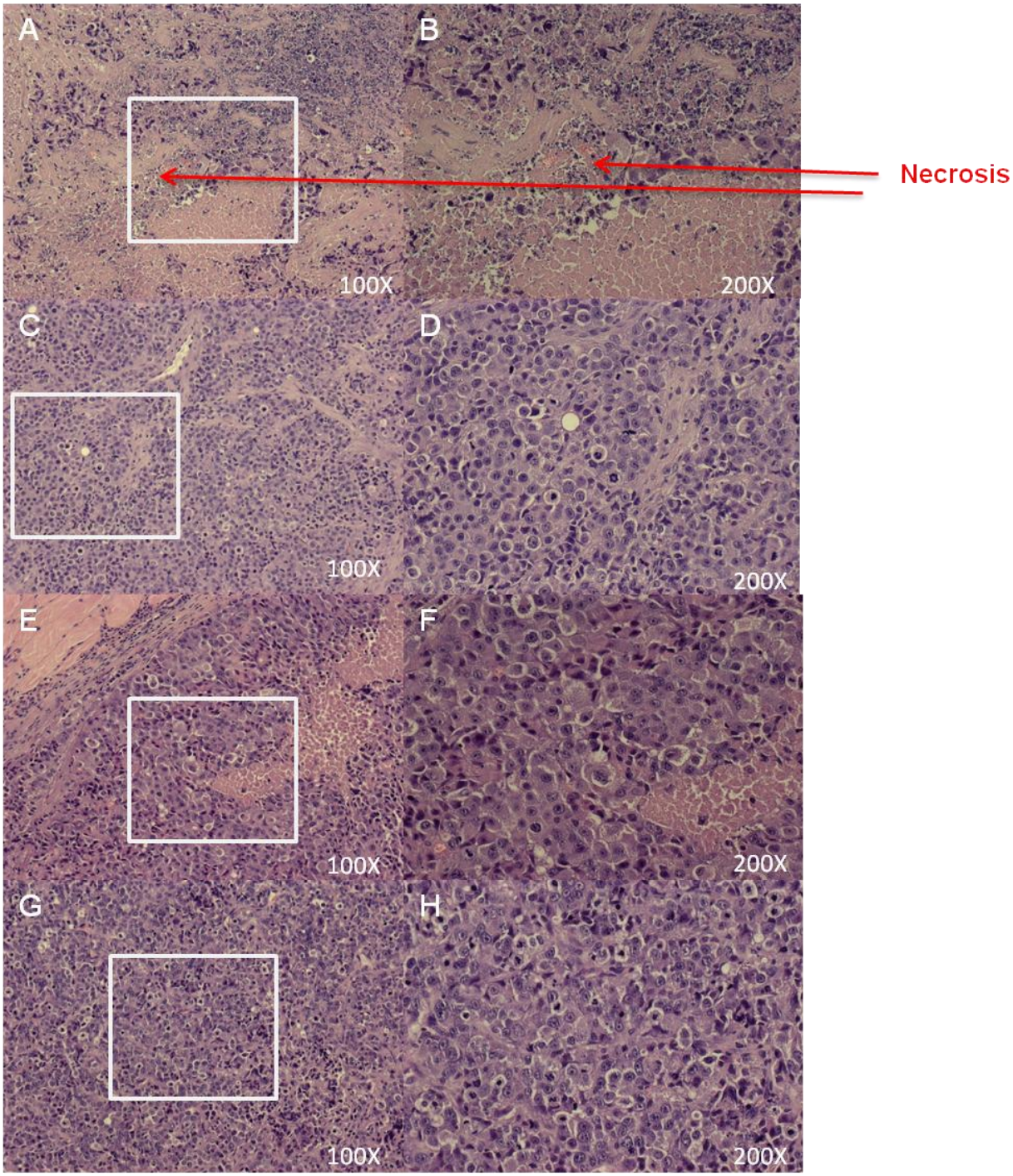


Figure 66 H&E staining of LNCaP-CDXR3 tumour xenografts.

LNCaP-CDXR3 tumour xenografts were fixed in 4% formalin for 24 h and stored in 70% ethanol. Tumours were paraffin embedded and sections cut and stained with H&E. Panels show tumours treated with Ad5-TV-CU in combination with 5-FC at **A** 100x and **B** 200x, Ad5-GFP in combination with 5-FC at **C** 100x and **D** 200x magnification, Ad5-GFP in combination with PBS at **E** 100x and **F** 200x magnification and tumours treated with Ad5-TV-CU in combination with PBS at **G** 100x and **H** 200x magnification. Evidence of necrosis could be seen in xenograft tissue treated with Ad5-TV-CU in combination with 5-FC. No other inflammatory markers were identified.

While the pilot study for efficacy of Ad5-TV-CU in LNCaP-CDXR3 tumour xenografts appeared promising on some levels, showing complete tumour eradication in one animal, whilst the control tumour continued to grow, the ambiguity and varied results in terms both of the length of time it took for tumours to establish and grow and the response to viral therapy from both Ad5-GFP and Ad5-TV-CU highlights the unreliability of this model in BALB/c male mice. A large number of animals would be required to generate sufficient significant results using this cell line, and would be too costly for further studies.

5.2.2 Pilot study in BALB/C mice with LNCaP-104-S tumour xenografts

5.2.2.1 Ad5-TV-CU in combination with 5-FC impeded relative tumour growth in two animals.

In the second pilot study, I aimed to investigate efficacy and safety of Ad5-TV-CU in combination with 5-FC *in vivo,* using LNCaP-104-S tumour xenografts in hormonally intact male BALB/c athymic mice. 7 male BALB/c athymic mice with 60-day slow release 1.25 mg testosterone pellets implanted under the skin (upon recommendation from Dr John Kokontis (Kokontis et al. 2005) were injected subcutaneously with $1x10^6$ hormone dependent LNCaP-104-S cells.

Palpable tumours were undetectable by week 5. Initially it was thought that the tumour cells died because the testosterone pellets were inserted on the same day as the cells were injected, not allowing sufficient time for release of the high levels of testosterone required to support growth of the LNCaP-104-S. Due to this, an additional inoculation with $2x10^6$ LNCaP-104-S cells in 50% matrigel was applied after 5 weeks into both flanks. Three weeks later tumours developed in one flank in four out of seven animals. The remaining animals did not develop tumours and were therefore culled. LNCaP-104-S tumour xenografts in two of four animals reached >100 mm^3 within 4 weeks, tumours on the remaining animals were <100 mm^3 on treatment day 1 (Figure 67A).

After allowing the tumours to grow for a further 4 weeks to ~100 mm^3 tumour xenografts were injected intratumourally with $1x10^{10}$vp of Ad5-TV-CU on days 1, 3 and 6, for a total of three doses. Animals were subsequently randomised to receive intraperitoneal injections of either 100 mg/kg 5-FC (red lines) or an equal volume of PBS (yellow lines) on days 2, 5, 8 and 15, totalling 4 doses, so that the

total treatment period was 15 days. As a result of poor tumour growth there were a total of two tumour xenografts in each treatment group (Ad5-TV-CU plus 5-FC and Ad5-TV-CU plus PBS). Treatment with Ad5-TV-CU in combination with 5-FC prevented increased tumour growth in both animals (Figure 67). However, in control treated animals (Ad5-TV-CU+PBS) only one tumour continued to grow up to day 35 after treatment initiation while the second tumour remained stable throughout the study.

Overall the LNCaP-104-S tumours were slow growing with only two out of six tumours reaching >200mm^3 by the time the study was terminated, 44 days after treatment initiation and the largest tumour did not exceed 600 mm^3. The animals were culled due to the protracted growth of the tumours (including controls), which were below the acceptable size limits for tumour burden (Home Office regulations) when the pilot study was terminated (Figure 67A+B). Three days after treatment was initiated, measureable tumours developed in the opposite flank of the two control animals receiving Ad5-TV-CU in combination with PBS. These tumours also presented variable growth, one untreated tumour continued to grow throughout the experiment, however growth of the second untreated tumour failed to reach >200mm^3 (Figure 67; purple lines). Additionally, a further 22 days after treatment was initiated, tumours developed in the opposing flank of the group of animals receiving Ad5-TV-CU in combination with 5-FC and continued to grow, albeit to <200 mm^3 until the animals were culled at day 44 (additional purple lines at day 22, Figure 67A).

There was a marked reduction in the average relative tumour growth over time in the group treated with Ad5-TV-CU in combination with 5-FC in comparison to both the control PBS group and the untreated tumour group up until day 35 (Figure 67A). Conversely, by day 44 the average tumour volume in the Ad5-TV-CU plus PBS group dropped. At this stage the study was terminated due to erratic tumour growth. The difference in relative tumour volume between the groups at day 35 was not significant, due to the huge variability in tumour size, the limited number of animals (n=2) and as a result the large standard error within the Ad5-TV-CU plus PBS treatment group and untreated controls (shown by error bars). Contrary to the large variability seen in control groups, treatment with Ad5-TV-CU in combination with 5-FC resulted in much less variability in tumour volume, and therefore a smaller standard error within the group, suggesting that a combination of Ad5-TV-CU and 5-FC results in consistent relative tumour growth inhibition.

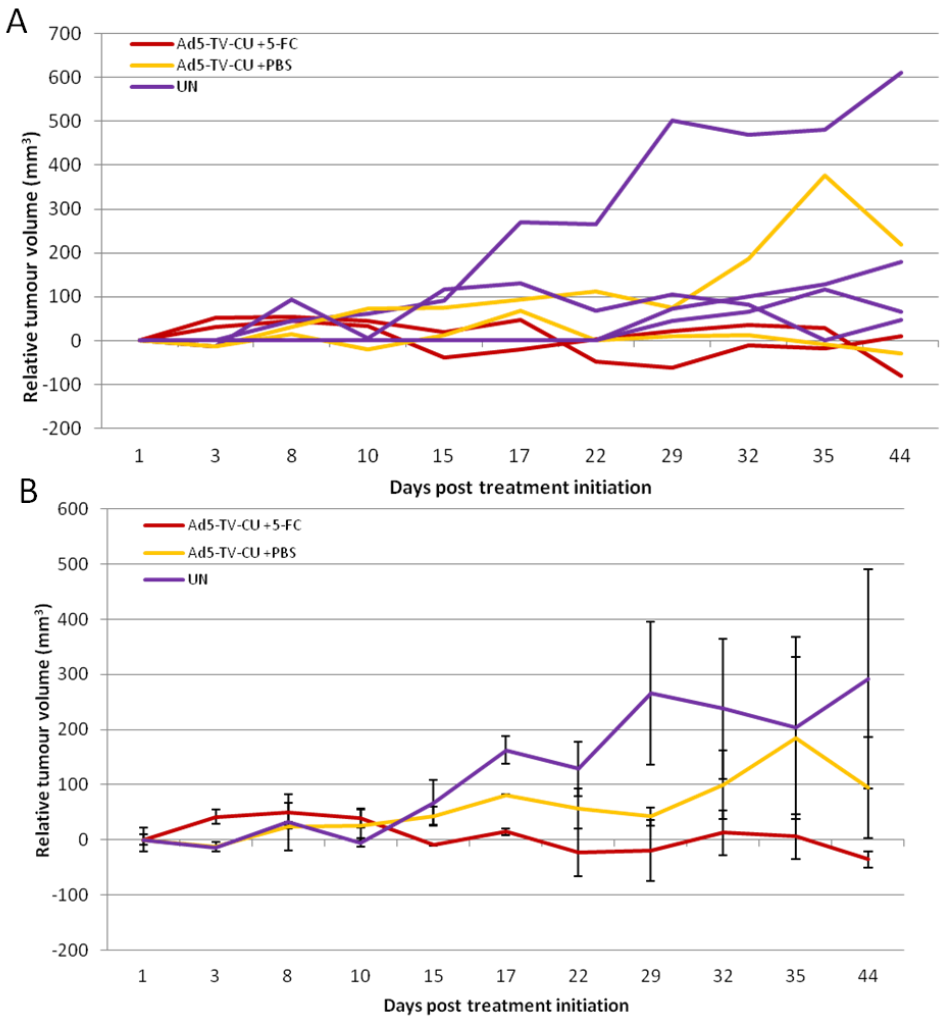


Figure 67 Average relative tumour volume of LNCaP-104-S xenografts remains the same with treatment of Ad5-TV-CU in combination with 5-FC, but increases in combination with PBS

Male BALB/c athymic mice with subcutaneous 60-day slow release 1.25 mg testosterone pellets were injected subcutaneously with hormone dependent LNCaP-104-S cells. After allowing the tumours to grow for 3 weeks to ~100 mm^3 mice were separated into control group (Ad5-TV-CU plus PBS (yellow line), or Ad5-TV-CU plus 5-FC (red line) and the time was designated as day 1. Tumours that developed a number of days after treatment began were left untreated (purple line). **A.** Data shows tumour growth post treatment initiation **B**. Data shows the average tumour volume measurements in mm^3 ±SEM over a total period of 44 days. Tumour volume was normalised relative to the individual starting tumour volume determined on day 1 of treatment, to illustrate the effect of treatment on relative tumour volume and averages plotted over 44 days ± SEM. n= number of tumours per group. n=2 (Ad5-TV-CU+5-FC, Ad5-TV-CU+PBS, UN) UN= untreated. p=>0.05 by one-way anova. Graphs showing matched tumours in each individual animal can be seen in the appendix section 8.2, Figure_Apx2.

5.2.2.2 Treatment with Ad5-TV-CU in combination with 5-FC prevented further tumour growth in one animal compared to treatment with 5-FC alone.

One animal bearing an LNCaP-104-S tumour on the right flank was injected with Ad5-TV-CU on days 1, 3 and 6. An intraperitoneal injection of 100 mg/kg 5-FC was given on days 2, 5, 8 and 15. Initially tumour volume decreased throughout the treatment period up to day 15 (last day of treatment) and then began to steadily rise again (Figure 68A). At day 22 a measurable tumour developed in the left flank of the animal and continued to grow until the pilot study was terminated. At day 35 there was a sudden reduction in tumour volume in the xenograft treated with Ad5-TV-CU in combination with 5-FC (Figure 68A). When dissected from the animal, the tumour treated with Ad5-TV-CU in combination with 5-FC was visibly smaller than the untreated tumour (Figure 68B).

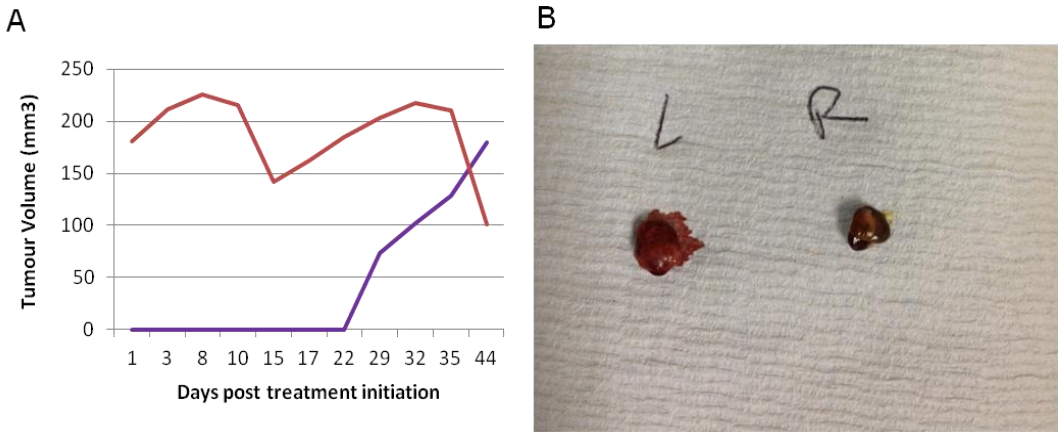


Figure 68 Combination of Ad5-TV-CU and 5-FC reduced relative tumour volume of LNCaP-104-S xenografts

A. Tumour volumes of two LNCaP-104-S tumour xenografts on the left and right flank of one animal, injected with 10^{10}vp Ad5-TV-CU in combination with 5-FC (red line), or left untreated with intraperitoneal (IP) injection of 5-FC (purple line). **B.** LNCaP-104-S tumour xenografts from one animal injected intratumourally with Ad5-TV-CU (R) or left untreated (L) in both with IP 5-FC injection.

5.2.2.3 H&E staining of LNCaP-104-S tumour xenografts highlights highly necrotic areas of the tumours

In order to better understand the poor tumour growth of LNCaP-104-S xenografts, H&E staining was performed on LNCaP-104-S xenograft tissue infected with Ad5-TV-CU in combination with either 5-FC (Figure 69A+B) or PBS (Figure 69C+D) or untreated control tumours (Figure 69E+F). There was little difference in the morphology of xenografts infected with Ad5-TV-CU in comparison to uninfected

xenografts (Figure 69A+C). All xenografts showed areas densely populated with erythrocytes and signs of necrosis (Figure 69A-D). On closer inspection there was also evidence of an inflammatory response, with Eosinophils and B-lymphocytes expressed in the xenografts (Figure 69D). It is possible that the inhibition of LNCaP-104-S tumour xenograft growth could be due to a combination of immune response initiated in the animals, as they get older, but is more likely due to the necrotic areas seen in the tumours or growth inhibition due to exorbitant levels of testosterone. Untreated tumours were highly populated with erythrocytes, but did not show evidence necrosis (Figure 69E-F).

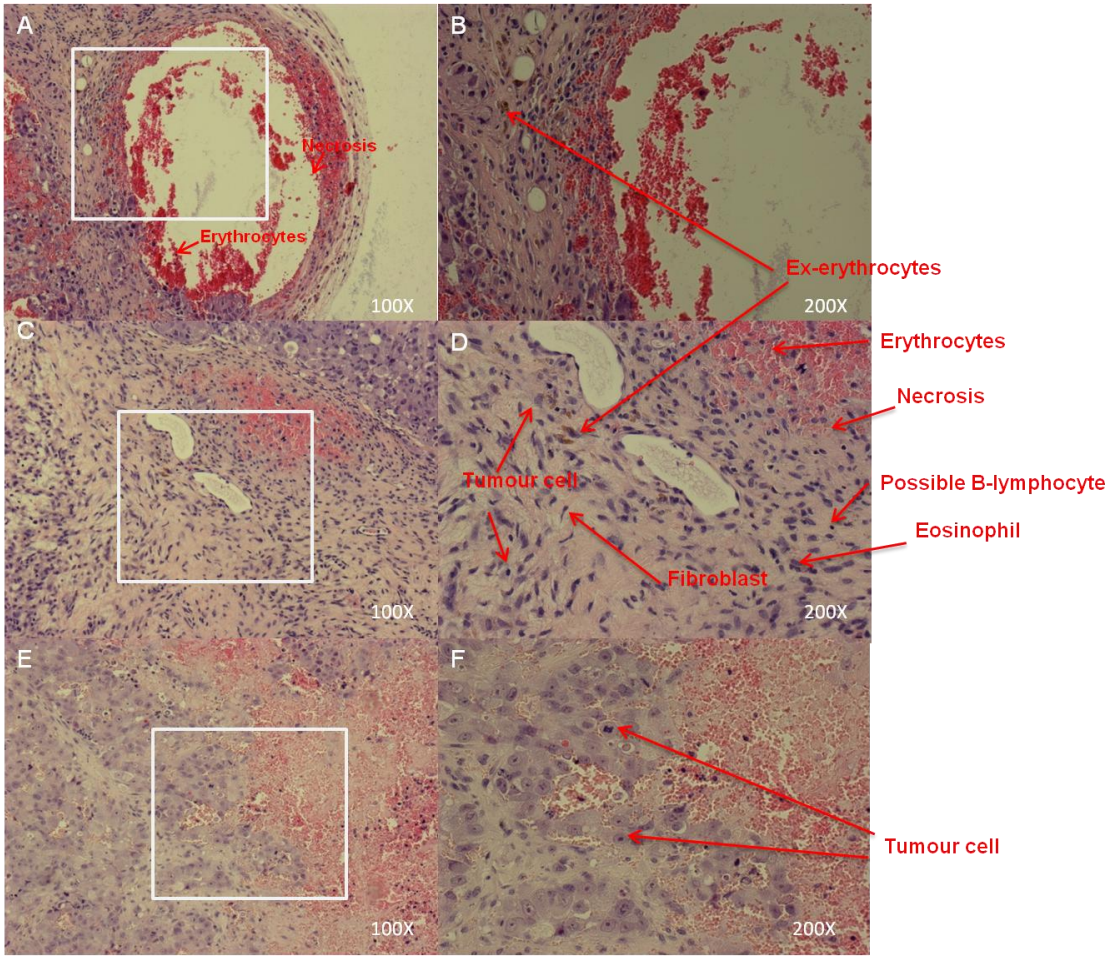


Figure 69 H&E staining of LNCaP-104-S tumour xenografts highlights necrotic tumours

LNCaP-104-S tumour xenografts were fixed in 4% formalin for 24 h and stored in 70% ethanol. Tumours were paraffin embedded and sections cut and stained with H&E. Panels show tumours from animals treated with Ad5-TV-CU in combination with intraperitoneal (I.P) doses of 5-FC at **A** 100x and **B** 200x magnification, tumours treated with Ad5-TV-CU in combination with PBS at **C** 100x, and **D** 200x magnification and untreated control tumours at **E** 100x and **F** 200x

5.2.3 Establishing a new animal model: NOD/SCID mice with LNCaP-104-S tumour xenografts

Upon further communication with Dr Kokontis it was suggested that the levels of testosterone in the mice with 1.25 mg testosterone pellets may in fact be inhibitory to the growth of the tumours, referring previous studies (Chuu et al. 2006). In order to establish whether the slow growth of LNCaP-104-S tumour xenografts in male BALB/c athymic mice was due to exorbitant levels of testosterone and inhibition by increasing levels of immune factors, I aimed to explore growth in animals that were severely immunodeficient (NOD/SCID; T and B cell development affected) with and without testosterone pellets. Six 10-week old male NOD/SCID athymic mice were separated into two groups. Three animals were treated as described above (60 day slow release subcutaneous 1.25 mg testosterone pellets) and the remaining three animals were left without additional testosterone, in order to establish a possible inhibitory effect on tumour volume by excess hormone. Two days later the animals were injected subcutaneously with either $2x10^6$ or $5x10^6$ LNCaP-104-S cells in the left and right flank respectively.

No measureable tumours developed in the animals bearing subcutaneous testosterone pellets, suggesting that the high levels of testosterone may in fact have a negative effect on the growth of LNCaP-104-S tumour xenografts. However, measureable tumours established 33 days after injection the animals without testosterone pellets but only developed in one flank. Interestingly, this did not correlate with the number of cells that were injected, as tumour growth between the left and right flank was random. One animal showed signs of poor health on day 14 after measurable tumours developed, and thus had to be culled. By day seven, after measurable tumours developed, tumour volume had plateaued in one animal (Figure 70), remaining the same size for a further three weeks. Tumours were measured twice weekly for one month. Tumour burden was well below the limit set by the Home Office before the study was terminated due to poor tumour growth.

Despite the fact that LNCaP-104-S tumour xenografts grew in animals without testosterone pellets the protracted growth of the tumours makes this model unreliable. As the tumours did not reach larger volumes, this is an unsuitable model for efficacy studies using Ad5-TV-CU, as it is impossible to determine

whether reduced tumour growth is due to treatment with Ad5-TV-CU or arbitrary growth of the tumours.

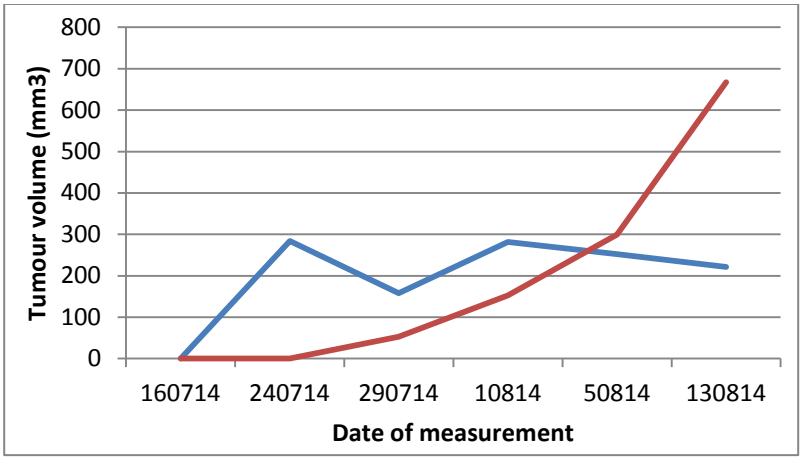


Figure 70 Growth of LNCaP-104-S tumours in NOD/SCID mice is unreliable

Three 10 week old male NOD/SCID mice bearing subcutaneous 1.25 mg testosterone pellets and 3 10 week old male NOD/SCID mice without pellets were injected subcutaneously with $2x10^6$ or $5x10^6$ hormone dependent LNCaP-104-S cells in the left or right flank respectively. Only the animals that did not have a subcutaneous testosterone pellet implanted grew tumours. Of which, two of the animals grew tumours from $2x10^6$ injected LNCaP-104-S cells and one animal grew a tumour from $5x10^6$ cells. Tumours were measured twice weekly. Data shows the tumour volume over time in mm^3 in two individual mice that developed tumours, the third animal had to be culled due to poor health.

5.2.4 Establishing a new fast growing tumour model, 22RV1 xenografts in CD-1 athymic mice.

5.2.4.1 Ad5-TV-CU impedes average relative tumour growth in animals treated in combination with 5-FC and PBS.

Following difficulties in establishing a reliable tumour model with both LNCaP-104-S and LNCaP-CDXR3, a new model was established in CD-1 athymic mice with 22RV1 cells. In this fourth pilot study, I aimed to investigate efficacy and safety of Ad5-TV-CU in combination with 5-FC *in vivo,* using 22RV1 tumour xenografts in intact male CD-1 athymic mice. Previous studies in the Halldén lab suggested that efficacy in response to replicating adenovirus mutants was difficult to achieve using the 22RV1 model because of the high cell proliferation rate (Unpublished studies). However, I speculated that the combination of Ad5-TV-CU with 5-FC could result in more efficient inhibition of tumour xenograft growth, due to the combination of virus and drug. To test this idea, twelve 6 week old male CD-1 athymic mice were inoculated with $1x10^6$ 22RV1 cells in 50% matrigel in the left

flank. Palpable tumours were detectable by day 12 and were injectable (~100 mm^3 calculated by W^2 x L x 0.52) by day 14.

One group of animals with larger tumours was left completely untreated in order to monitor 22RV1 tumour growth. The 22RV1 tumour xenografts were injected intratumourally with 10^{10}vp of Ad5-TV-CU in the left flank on days 1, 3 and 6, for a total of three doses. The animals were subsequently randomised into groups that received an intraperitoneal injection of either 5-FC at 100 mg/kg or an equal volume of PBS on days 2, 5, 8 and 15. Tumour volume was monitored twice weekly. Treatment continued until day 15. By this time all untreated animals were culled due to excessive tumour burden.

There were a total of four 22RV1 xenografts in each treatment group (Ad5-TV-CU plus 5-FC and AD5-TV-CU plus PBS and untreated). Most surprisingly, the combination of Ad5-TV-CU with PBS appeared to have the most striking effect on tumour volume. Two of the tumours treated with Ad5-TV-CU in combination with PBS failed to grow bigger than 250 mm^3 up to day 32 post treatment, and those animals were culled due to the poor tumour growth. Although these tumours were relatively small upon treatment initiation (~70 mm^3), they were comparable to the starting volume of the tumours treated with Ad5-TV-CU in combination with 5-FC (see appendix section 8.2, Figure_Apx3). 22RV1 tumours grew at a fast rate, with tumours on seven of twelve animals reaching critical limits shortly before or just after treatment finished on day 15. This accelerated growth may prevent any real differences between the control groups and the group treated with Ad5-TV-CU to be identified.

Overall there was a marked reduction in the average relative tumour volume over time in both groups treated with Ad5-TV-CU in combination with 5-FC or PBS in comparison to the untreated tumour group up until day 14 when the first untreated control was culled (Figure 71). The difference in relative tumour volume between the groups at day 14 is not significant, due to the huge variability in tumour size, the limited number of animals (n=4) and as a result the large standard error within the groups (shown by error bars). The slow growth rate of the tumours treated with Ad5-TV-CU in combination either 5-FC or PBS suggests that manipulation of the tumour may attenuate its growth, or that Ad5-TV-CU alone can slow the growth rate of 22RV1 tumour xenografts, regardless of whether it is or is not administered in combination with the 5-FC prodrug.

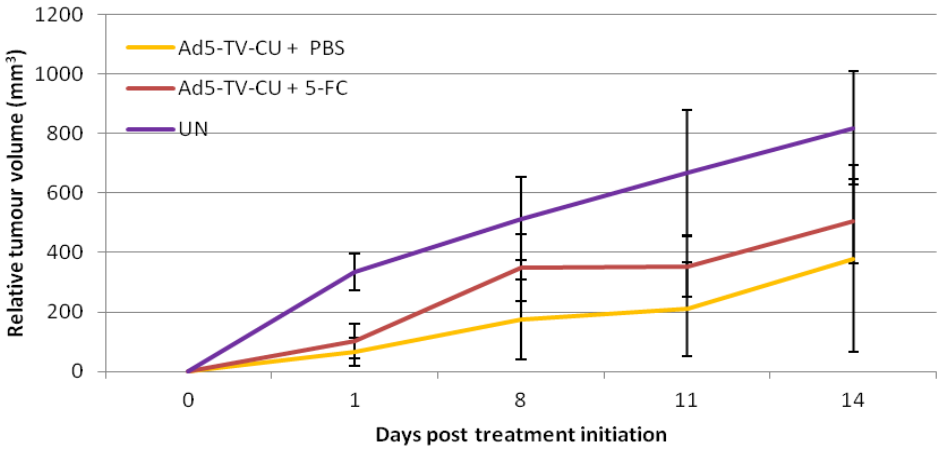


Figure 71 22RV1 xenografts treated with Ad5-TV-CU in combination with 5-FC or PBS grow at a slower rate in comparison to the untreated control

Male CD-1 athymic mice were injected subcutaneously with $1x10^6$ hormone independent 22RV1 cells. After allowing the tumours to grow for 2 weeks to ~100 mm^3 the mice were separated into control groups (Untreated (purple line) and Ad5-TV-CU plus PBS (yellow line) and a target group Ad5-TV-CU plus 5-FC (red line) and the time was designated as day 1. Data shows the average tumour volume measurements in mm^3 ±SEM up until the first mouse was culled on day 14. Tumour volume was normalised relative to the individual starting tumour volume determined on day 1 of treatment to illustrate the effect of treatment on relative tumour volume. n= number of tumours per group. n=4 (Ad5-TV-CU+5-FC, Ad5-TV-CU+PBS, UN) UN= untreated $p>0.05$ determined by one-way anova.

5.2.4.2 H&E staining of 22RV1 tumour xenografts

H&E staining was performed on 22RV1 xenograft tissue infected with Ad5-TV-CU in combination with 5-FC, PBS or left untreated (Figure 72). There was little difference in the structure of xenografts infected with Ad5-TV-CU in comparison to uninfected xenografts (Figure 72). Both treated and untreated tumours showed areas highly populated with erythrocytes (Figure 72A-F). On closer inspection there was also evidence of lymphocyte infiltration (Figure 72B).

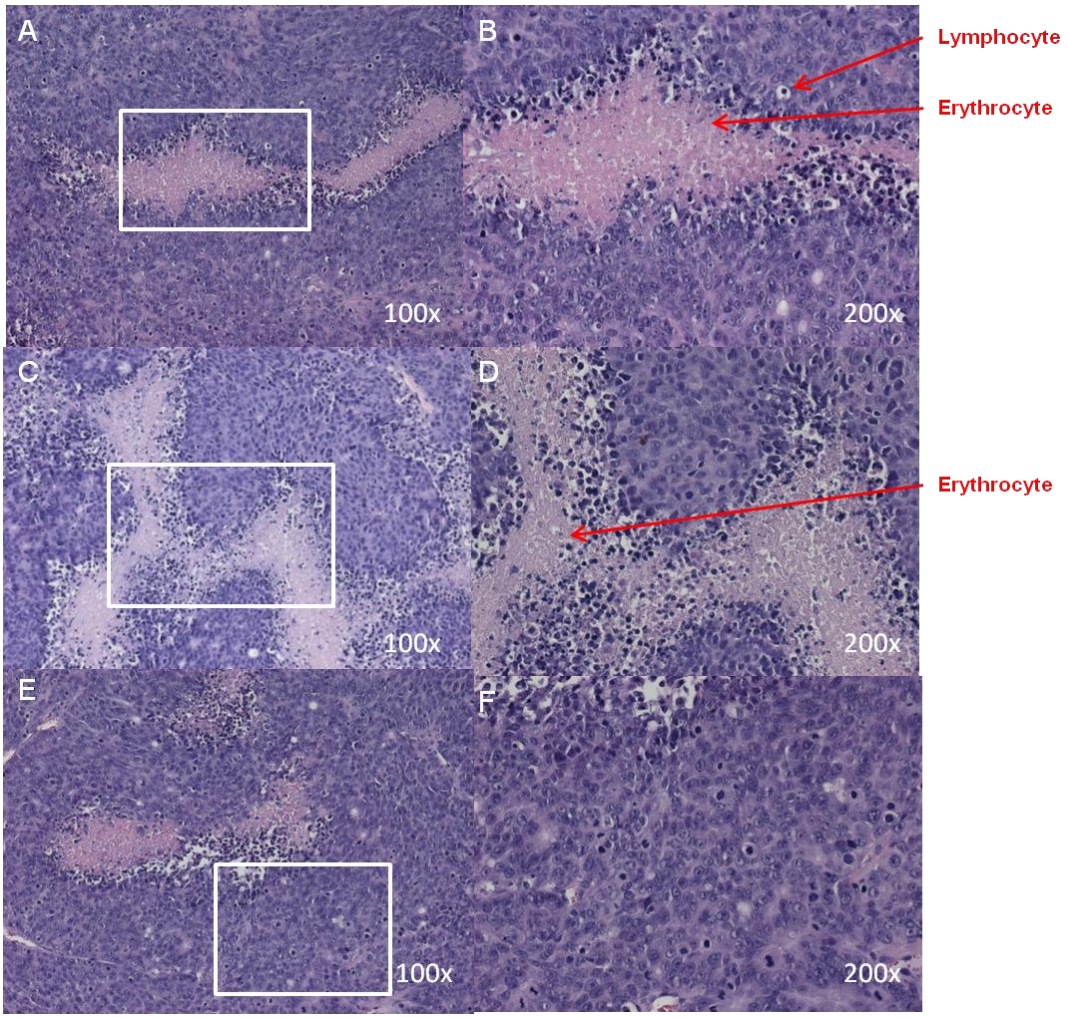


Figure 72 H & E staining of 22RV1 treated xenografts treated with Ad5-TV-CU in combination with 5-FC, PBS or untreated

22RV1 tumour xenografts were fixed in 4% formalin for 24 h and stored in 70% ethanol. Tumours were paraffin embedded and sections cut and stained with H&E. Panels show tumours treated with Ad5-TV-CU in combination with I.P dose of 5-FC at **A** 100x and **B** 200x magnification, tumours treated with Ad5-TV-CU in combination with PBS at **C** 100x and **D** 200x magnification and untreated control tumours at **E** 100x and **F** 200x.

In summary four pilot studies were performed with a range of different animal and tumour models to try and establish an effective model with which to test the Ad5-TV-CU virus. Of these, reliable tumour growth could not be established in three out of four models (LNCaP-CDXR3 and LNCaP-104-S in BALB/c and LNCaP-104-S in NOD/SCID mice), and the tumours grew too rapidly in the final model (22RV1 in CD-1 mice), resulting in unreliable results. These are summarised below in Table 31.

Table 31 Summary of *in vivo* experiments performed with Ad5-TV-CU

Cell line	Animal Model	Special treatment	Outcome
$1x10^6$ LNCaP-CDXR3 in 50% matrigel	BALB/c	Castrated male mice	Poor tumour growth- majority of tumours plateaued at 200 mm^3 Trend towards decreased tumour volume with Ad5-TV-CU in combination with 5-FC compared to combination with PBS
$1x10^6$ LNCaP-104-S in 50% matrigel	BALB/c	Intact male mice with subcutaneous 1.25 mg testosterone pellet	Poor tumour growth- majority of tumours plateaued at 200 mm^3. Trend towards decreased tumour volume with
$2x10^6$ LNCaP-104-S in 50% matrigel	NOD/SCID	Half intact male mice with subcutaneous 1.25 mg testosterone pellet, half without	Xenografts did not grow in mice with testosterone pellets but grew irregularly in animals without testosterone pellets
$1x10^6$ 22RV1 in 50% matrigel	CD-1	Intact male mice	Rapid tumour growth, but no difference between tumours treated with Ad5-TV-CU in combination with 5-FC and Ad5-TV-CU in combination with PBS

5.3 DISCUSSION

There were difficulties establishing a reliable *in vivo* model with which to test Ad5-TV-CU. There were a number of potential reasons for this, dependent on both the cell type and the animal model.

For LNCaP-CDXR3 and LNCaP-104-S xenografts that were successfully injected, it was difficult to establish if there was a difference between the treatment of tumours with Ad5-GFP and Ad5-TV-CU. Most disappointingly, the same trend of relative tumour volume inhibition was seen in tumours treated with either Ad5-TV-CU or Ad5-GFP in combination with both 5-FC and PBS in LNCaP-CDXR3 tumours. While this data was unexpected, there was little variation in tumour volume between the groups, as shown by small standard errors. This small variation could be due to one or more of three things: 1) the tumours reached their maximum size and were therefore unable to grow further, due to limitations such as blood supply 2) Injection with Ad5-GFP alone had off target toxicity in LNCaP-CDXR3 xenografts 3) An immune response was activated in reaction to viral infection and prevented relative tumour growth. Graphs representing each individual animal can be found in the appendix section 8.2.

Previous studies reported that growth of androgen-dependent LNCaP sublines in mice required the addition of testosterone for successful tumour growth (Umekita et al. 1996) and we were advised upon receipt of the cells (by Dr Kokontis and colleagues) that this would be the best course of action to enable xenograft growth. Additionally, BALB/c athymic mice have highly reduced levels of testosterone in comparison with their immunocompetent littermates, therefore testosterone pellets were inserted under the skin of hormonally intact BALB/c male athymic mice to support the growth of LNCaP-104-S xenografts. Consequently, it is possible that exorbitant levels of testosterone in the animals due to their age (15 weeks, as the tumours took longer than expected to establish), combined with the implantation of the testosterone pellets, resulted in erratic and inhibitory growth of the tumours. In fact, LNCaP cells have previously been shown to respond within a narrow range of 10^{-11} to 10^{-9} M DHT with 10^{-7} M causing cell growth inhibition (Gregory, Johnson, et al. 2001).

Contrarily, LNCaP-CDXR3 cells are an androgen-independent cell line, generated from the parental LNCaP-104-S cell line (section 5.1), requiring castrated male mice for tumour growth *in vivo*. Previous studies by Kokontis et al have shown that introduction of androgens in male mice bearing LNCaP-CDXR3 xenografts results in complete tumour regression in 70% of animals (Chuu, Kokontis et al. 2011). One possible explanation for the varied tumour growth results in our pilot study could be residual levels of circulating androgens in the male BALB/c mice (from incomplete orchiectomy). Despite orchiectomy, it is also possible that extratesticular androgens were produced due to the older age of the animals. One way to determine this in future studies would be by quantifying serum PSA levels in the animals or by measuring testosterone/DHT levels.

It is also possible that as the animals aged there was an elevated immune response to LNCaP-CDXR3 and LNCaP-104-S tumour xenografts, which contributed to the inhibition of tumour growth. In fact, H&E staining of LNCaP-104-S tumour xenograft sections revealed evidence of immune cell infiltration. BALB/c athymic mice lack the thymus, resulting in the absence of T-lymphocytes from thymus-dependent areas of both lymph nodes and spleen and a reduced lymphocyte population, comprised almost entirely of B-cells. This immunodeficiency means they are unable to reject xenogeneic skin and tumour grafts and therefore make them an ideal model for establishing human tumour xenografts. It is known that in older athymic mice, T-cell maturation in an extrathymic environment (from spleen and lymph nodes) increases and the proportion of CD4+ and CD8+ T-cells increases (Kennedy, Pierce, and Lake 1992). This results in the accumulation of increasing numbers of lymphocytes that could have a detrimental effect on LNCaP-CDXR3 tumour xenograft growth. However, the animals were only 20 weeks old when culled so this may not have had as much of an effect as the response to viral infection as anticipated due to their young age.

Additionally, the LNCaP-104-S tumour xenografts that developed were highly vascularised, with a black appearance under the skin. When the animals were culled and the tumours dissected, there was clear evidence of neovascularisation and tumours were full of blood. This could also be seen by the highly dense areas of erythrocytes in H&E stained sections combined with areas of necrosis in treated LNCaP-104-S tumours. It is therefore fair to conclude that the reason for the slow growth of LNCaP-104-S tumours cannot be explained by poor blood supply.

Neovascularisation was not seen at such high levels in the castration resistant subline LNCaP-CDXR3, supporting a potential role of high levels of testosterone in the promotion of angiogenesis in these tumours. In fact, previous studies have suggested that testosterone-replacement therapy is associated with enhanced expression of HIF-1a, SDF-1a and VEGF and promotes angiogenesis after acute myocardial infarction in castrated rats (Chen et al. 2012).

The poor growth of control LNCaP-104-S and LNCaP-CDXR3 tumours treated with Ad5-GFP in combination with both PBS and 5-FC suggests that Ad5-GFP is intrinsically toxic to these xenografts, or that tumours are very sensitive to physical manipulation. However, this would be difficult to investigate, as it would require tumours to be injected with PBS alone according to the same schedule as the virus; this would not be recommended due to the 3R's policy, aim at reducing the number of animals used for *in vivo* studies. I previously demonstrated that a combination of Ad5-GFP and 5-FC did not enhance cell killing in LNCaP-CDXR3 and LNCaP-104-S cells grown *in vitro* (section 4.2.4.3), however Ad5-GFP alone is intrinsically toxic in these cells (LNCaP-CDXR3 EC_{50} 3707 ± 1174 ppc and LNCaP-104-S EC_{50} 2245 ± 423 ppc). Nevertheless, it is difficult to conclude that intrinsic toxicity is responsible for poor tumour growth in this treatment group, as this could be due to the slow growing nature of the cell lines in BALB/c mice. In fact, one of the LNCaP-CDXR3 tumours did not develop until 10 weeks after subcutaneous injection of tumour cells, and LNCaP-CDXR3 tumour xenografts failed to grow in one animal. LNCaP-104-S tumours developed in only four of seven animals in one flank, with the other flank developing weeks later. The protracted development of these tumours demonstrates the slow growing nature of LNCaP-CDXR3 and LNCaP-104-S xenografts and exemplifies their unreliability for use in *in vivo* studies, as it was impossible to truly determine whether Ad5-TV-CU/Ad5-GFP is responsible for causing reduced tumour volume, rather than the absence of tumour growth, once a small initial increase in tumour volume was reached. What could be established from the Ad5-GFP control, is that there is no difference in relative tumour growth between xenografts treated with a combination of Ad5-GFP and 5-FC or Ad5-GFP alone, in agreement with my observations *in vitro*. If these studies were to be repeated, a dose response to 5-FC in the presence of Ad5-TV-CU and GFP should clearly show increased efficacy only with Ad5-TV-CU but will, of course, only work if the tumours are growing.

The poor growth rate of LNCaP-CDXR3 and LNCaP-104-S tumours in Male BALB/c athymic mice could be due to a number of reasons, for example the age of the animals, their immune responses and inhibition by testosterone. Efforts to eliminate some of these variables when working with LNCaP-104-S tumour xenografts by using more immunodeficient mice (NOD/SCID, deficient in T-cells and B-cells) or injecting LNCaP-104-S cells in mice without subcutaneous testosterone pellets were also unsuccessful. Previous studies by Chuu et al showed that testosterone levels had no impact on LNCaP-104-S tumour xenograft growth (Chuu, Hiipakka et al. 2006). However, results from our pilot study in NOD/SCID athymic mice showed that, whilst LNCaP-104-S xenografts grew in NOD/SCID mice that did not have a subcutaneous testosterone pellet, tumours did not grow in animals with subcutaneous testosterone pellets, suggesting that testosterone pellets had an inhibitory effect on the growth of LNCaP-104-S tumours *in vivo.* This evidence, combined with increased neovascularisation of LNCaP-104-S tumour xenografts, suggests response to testosterone is more complex in this cell line than previously thought, and is different to other LNCaP sublines that are dependent on testosterone for tumour take. Thus, high levels of testosterone prevent LNCaP-104-S tumour development in NOD/SCID athymic mice. This is potentially the inhibitory factor in LNCaP-104-S xenograft growth in the initial pilot study. Despite this discovery, LNCaP-104-S relative tumour growth was still unpredictable in mice without subcutaneous testosterone pellets, with varying growth patterns from animal to animal and it is consequently not an appropriate model. Despite difficulties in establishing tumours and inhibiting tumour growth in both pilot studies, treatment with Ad5-TV-CU in combination with 5-FC did show relative tumour growth inhibition in comparison to Ad5-TV-CU in combination with PBS in LNCaP-104-S and LNCaP-CDXR3. Whilst these results seemed promising, they were not significant in either study. In order to obtain significant results, an unreasonably large number of animals would be required. Therefore neither the LNCaP-104-S or LNCaP-CDXR3 cells are a reliable tumour model for use in larger *in vivo* studies.

Due to difficulties in utilising the LNCaP sublines for *in vivo* studies, 22RV1 androgen-independent xenografts were investigated, as they had previously demonstrated a high degree of cell killing with Ad5-TV-CU in combination with 5-FC (section 4.2.1.4). Injectable tumours were established within two weeks, a marked improvement on 4-6 weeks that the LNCaP sublines took to establish and

twelve out of fourteen of the tumours continued to grow until they reached the critical limit assigned by the Home Office (1.44 cm^2). Unlike the LNCaP sublines, the untreated 22RV1 tumours continued to grow to critical limits, demonstrating the reliability of 22RV1 xenograft growth. Thus, it could be concluded that any restriction on tumour growth was due to the treatments. 22RV1 xenografts continued to grow in all treatment groups throughout the duration of the experiment at a much faster rate than the LNCaP sublines. However, average tumour growth was restricted upon injection of Ad5-TV-CU in combination with 5-FC and PBS, though this was not significant due to large variation in the data. In fact, the combination of Ad5-TV-CU and PBS appeared to have a larger effect on restricting tumour growth than the combination with 5-FC. Whilst this was surprising to see, the result could be explained by two of the xenografts in the PBS control group that failed to reach >250 mm^3, even after all the other animals were culled due to tumour burden. This could possibly be due to the size of the tumour upon treatment initiation (both tumours were <100 mm^3 on treatment day 1), perforation of the tumour alone could be responsible for the poor growth, although tumours in the Ad5-TV-CU + 5-FC group, that were also of a similar size upon treatment initiation, continued to grow. Despite this, the majority of 22RV1 xenografts grew at a rapid rate with 10 out of 12 of the mice culled shortly after the treatment period ended (by day 25, 10 days after final treatment on day 15). This accelerated growth may prevent a real treatment effect being seen. As mentioned previously, the Halldén lab was unable to prevent the growth of 22RV1 xenografts with a replicating virus (unpublished data), due to accelerated tumour growth. Taken together, these findings suggest that despite potent CD/UPRT gene expression from the replication-deficient Ad5-TV-CU, the virus could not spread within the rapidly proliferating tumour mass and even in combination with 5-FC only a fraction of the tumour cells were eliminated. Furthermore, H&E staining of 22RV1 xenografts revealed dense areas of erythrocytes, suggestive of neovascularisation and efficient tumour blood supply with no obvious signs of necrosis.

In conclusion, we have still not established a suitable model for testing Ad5-TV-CU *in vivo;* the LNCaP sublines, LNCaP-CDXR3 and LNCaP-104-S failed to reliably grow. Most importantly, the androgen-dependent LNCaP-104-S cells would not grow efficiently with the addition of 1.25 mg slow release testosterone pellets, which is contradictory to previous *in vivo* studies that utilised other androgen-

dependent cell lines. The difficulty in establishing these xenografts meant that tumour volume measurements after treatment could not confirm whether tumour growth inhibition was due to treatment, or unreliable tumour growth. Whilst 22RV1 xenografts demonstrated more promising results, with continued tumour growth in 10 out of 12 animals there was still no clear difference between the groups treated with Ad5-TV-CU in combination with 5-FC compared to PBS. In order to generate significant results for Ad5-TV-CU treatment in combination with 5-FC in any of these animal models the number of animals that would be required, would not be in accordance with the Home Office 3R regulations. Therefore, identification of novel AR expressing PCa lines, patient ex vivo tissue specimens or an alternative model system for example zebrafish should be investigated to fully elucidate the effect of Ad5-TV-CU in combination with 5-FC *in vivo*.

CHAPTER SIX

IDENTIFYING THE OPTIMAL CHIMERIC TMPRSS2 PROMOTER FOR EFFICIENT ENZYME-PRODRUG THERAPY OF PCA

6.1 INTRODUCTION

Two of the most well characterised prostate specific genes are PSA and prostate specific membrane antigen (PSMA) (O'Keefe et al. 1998, Young et al. 1992a, Balk, Ko, and Bubley 2003a). PSA is currently used as a biomarker for PCa diagnosis, as elevated levels suggest the presence of PCa. As a result of this PSA has been extensively investigated for use as a prostate tissue specific promoter for PCa therapy (Segawa et al. 1998, Latham et al. 2000, Wu, Matherly, et al. 2001). However, due to poor levels of transgene expression that are obtained by using tissue specific promoters, there is still no widely used and effective tissue specific therapy for PCa.

Producing chimeric promoters has been one of the most successful methods of increasing transgene expression, often including the use of enhancers in combination with a tissue specific promoter. Previous studies had identified a 6 kb regulatory region of PSA, including the promoter and upstream regions (Schuur et al. 1996). Latham et al went on to demonstrate that a 1.6 kb enhancer region upstream of the PSA promoter was able to drive maximal transcriptional activity, in comparison to the entire 6 kb regulatory region of PSA (Latham et al. 2000). By combining the upstream 1.6 kb PSA enhancer sequence with either PSA or Kallikrein (Hklk2) promoters, transgene expression was increased 20-fold in comparison to the PSA promoter alone, in the PSA-positive cell line LNCaP, but not in PSA negative cell lines. Other groups have also used the PSA promoter in combination with enhancers to increase transgene expression. Wu et al were able to increase luciferase transgene expression 18.9-fold when duplicating the core PSA enhancer element in combination with the PSA promoter, in comparison to a single core PSA enhancer element with the PSA promoter (Latham et al. 2000, Wu, Matherly, et al. 2001, Sato et al. 2003). Sato et al achieved 20-fold higher expression compared to the native PSA promoter and enhancer construct, incorporation of this optimal chimeric promoter and enhancer into a TSTA system increased transgene expression by 1000-fold (Sato et al. 2003). Furthermore, the duplicated enhancer has been used in a number of adenoviruses to drive prostate specific expression of a reporter gene. Zhang et al found that incorporation of the duplicated enhancer into a TSTA system in an adenoviral vector drove 20-fold higher levels of luciferase expression than the CMV promoter (Zhang, Adams, et

al. 2002). However, many of these vectors suffered from poor efficacy in further studies.

Previous studies have identified the *TMPRSS2* promoter as an androgen regulated, prostate specific promoter (Lin et al. 1999, Lucas et al. 2008). Fusion of the promoter region of *TMPRSS2* and *ERG* functional sequence results in overexpression of *ERG* gene, which occurs in greater than 50% of PCa cases and is the most common fusion gene in human cancer (Mosquera et al. 2007). It has been shown that the fusion may promote malignant cell survival and proliferation (Demichelis et al. 2007). There are no other prostate specific promoters that frequently form fusion genes. We therefore hypothesised that, a specific element to the *TMPRSS2* promoter allows exceptionally high levels of oncogenic expression in comparison to any other previously validated prostate specific promoter, for example PSA. This promoter may therefore allow us to drive higher transgene levels than any other prostate specific promoters. We therefore set out to compare the promoter capabilities of TMPRSS2, including the L region (present in Ad5-TV-CU) and the original W region (discussed earlier), to the PSA promoter and the chimeric PSA promoter/enhancer, through evaluation of luciferase transgene expression.

6.2 RESULTS

6.2.1 TMPRSS2 promoter regions drive higher levels of prostate specific expression in the VISA system than the previously validated PSA promoter.

In order to further validate the use of the *TMPRSS2* promoter, the *PSA* promoter alone and the PSA promoter and enhancer (pDRIVE-PSA-hPSA, Invivogen), that have previously been demonstrated to drive high levels of prostate specific expression (Latham et al. 2000)(Figure 73A), were cloned into the original L-VISA plasmid in place of the L-ARE of TMPRSS2 to compare potency of the two promoter and enhancer systems (Figure 73B).

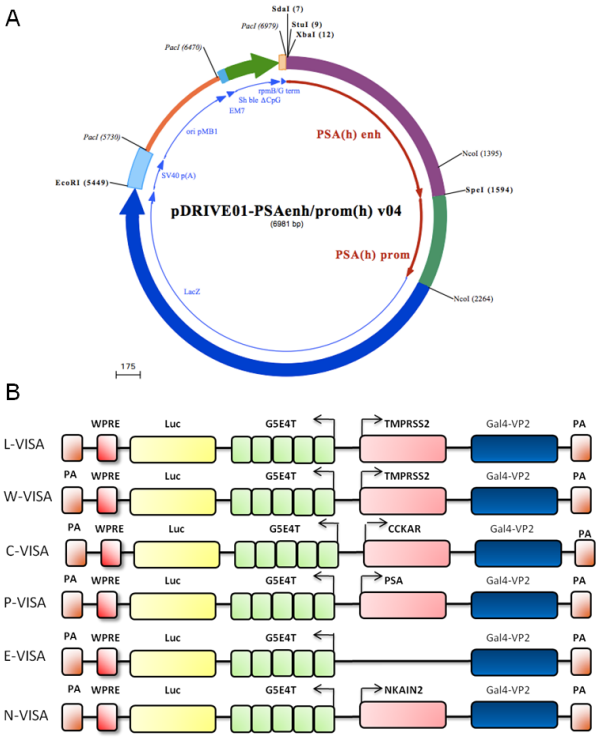


Figure 73 pDRIVE PSAenh/prom plasmid map purchased from Invivogen and VISA plasmids used in this study

A. The PSA promoter (green) was cloned into the empty VISA vector alone, or in combination with the PSA enhancer (purple) prom = promoter enh= enhancer **B**. Diagrammatic of VISA luciferase plasmids used in this study. Each system contains a Gal4-VP2 domain downstream of the specific promoter. Binding of the Gal4-VP16 fusion protein product to five Gal4 binding sites upstream of the Luciferase reporter gene results in targeted luciferase expression. Each construct contains a different promoter. L-VISA contains the L-ARE of TMPRSS2, W-VISA contains the W-ARE of TMPRSS2, C-VISA contains the CCKAR promoter, P-VISA contains the PSA promoter, E-VISA does not contain a promoter and finally N-VISA contains the NKAIN2 promoter.

The different promoter constructs coupled to the VISA-TSTA system, expressing luciferase as a reporter gene were transfected into three AR-positive cell lines, VCaP, LNCaP and 22RV1 and one AR-negative cell line, Panc1. Relative luciferase expression was determined using pRL (a plasmid expressing renilla luciferase under the control of SV40) as a control for transfection. The levels of expression from the plasmids were compared to the levels of that from the control E-VISA plasmid, which does not contain a promoter. The ideal vector for tissue specific promoter gene therapy must be both specific to the tissue, whilst driving high levels of expression of the transgene.

L-VISA activity was significantly high in 22RV1 cells, driving 76-fold more luciferase expression in comparison to the E-VISA control. However, there was no activity of this promoter in LNCaP cells and only 7-fold more luciferase expression in VCaP cells than E-VISA. Furthermore, there was no activity of L-VISA in Panc1 cells, demonstrating the specificity of this promoter. The level of expression from the W-VISA construct (based on a paper by Wang, containing a 1,011 base sequence located upstream from *TMPRSS2* Exon1) was 12076-fold higher compared with E-VISA in LNCaP cells (Figure 74). This was followed by VCaP cells, in which W-VISA drove 196-fold higher levels of luciferase in comparison to E-VISA. Finally, 22RV1 cells exhibited 126-fold higher levels of luciferase from W-VISA compared to the E-VISA. This was a smaller difference than VCaP because basal levels of luciferase in the E-VISA were much higher in 22RV1 cells than in VCaP cells. However, despite the promising results from W-VISA in AR-positive cell lines, there was no clear tissue specificity, as similarly high levels of luciferase expression from the W-VISA plasmid were seen in Panc1 cells.

Initially the activity of the original VISA plasmids was compared with that of the P-VISA plasmid, to establish whether the *TMPRSS2* promoter in L-VISA was capable of driving higher levels of transgene expression than the PSA promoter. Interestingly, the PSA promoter did not appear to demonstrate prostate specificity, and similar levels (~10-fold) of luciferase expression were seen in all four P-VISA transfected cell lines, including AR-positive 22RV1, VCaP and LNCaP and AR-negative Panc1 cells. Additionally P-VISA drove 26-fold, 610-fold, 268-fold and 18-fold higher levels of expression than E-VISA in 22RV1, LNCaP, Panc1 and VCaP respectively. This decreased specificity is similar to what is seen after transfection with W-VISA. Regardless of this the specific L-VISA vector is still able to drive 3-

fold higher levels of luciferase in comparison to P-VISA, suggesting that it is a stronger, more specific promoter.

The CCKAR (cholecystokinin type A receptor) promoter, a pancreatic-cancer-specific promoter that was originally received as the C-VISA vector from the MD Anderson centre, was included as a control for promoter activity in Panc1 cells. There was no pancreatic cancer specific transgene expression when C-VISA was transfected into the same panel of cell lines. In fact, luciferase expression from C-VISA was 2-fold higher in 22RV1 cells than Panc1, despite sequencing confirming that the CCKAR promoter was present. Additionally, another promoter for a non-prostate specific gene, NKAIN2 was cloned into the VISA vector as a control and luciferase expression levels quantified in 22RV1, HEK293, Panc1 and VCaP cells. NKAIN2 is not regulated by AR; however, luciferase expression levels driven by this promoter are 2-fold higher in the AR dependent prostate cell line 22RV1 in comparison to non-prostate cell lines, similar to what is seen with the C-VISA (Figure 74).

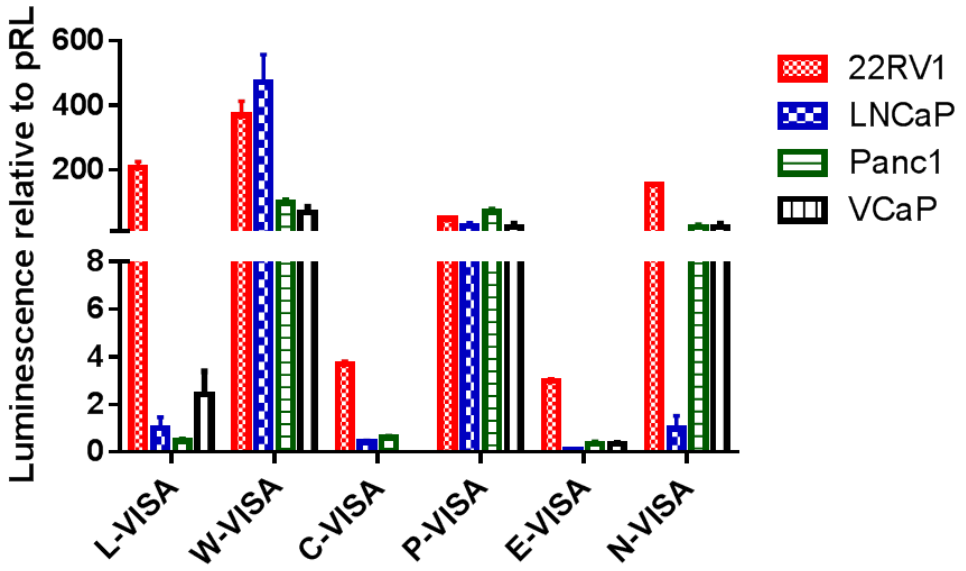


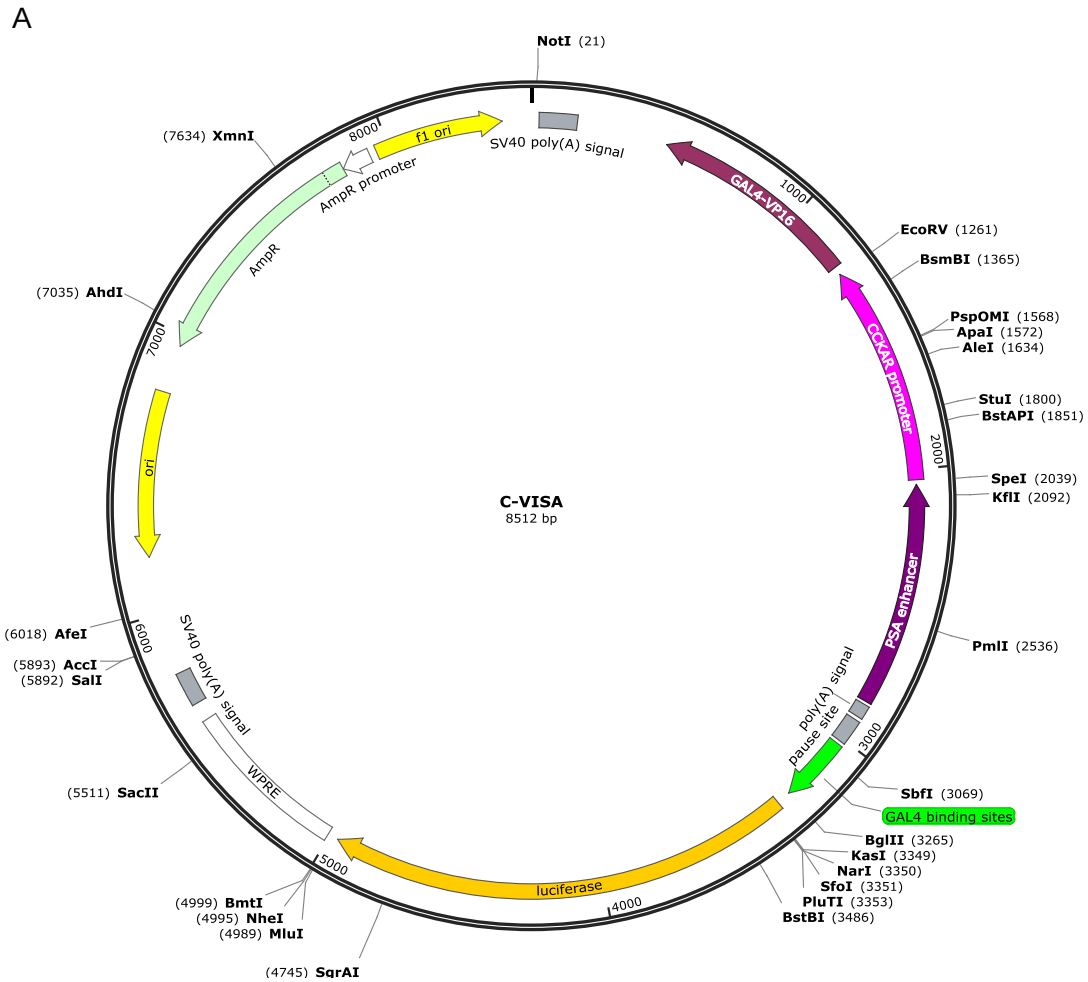
Figure 74 W-VISA is a much stronger promoter of luciferase expression but is not specific to AR-positive cell lines

$2x10^4$ 22RV1, LNCaP, Panc1 or VCaP cells were seeded and co-transfected the following day with 150 ng per well of the 6 different plasmids and 15 ng pRL-SV40 per well for normalisation, using lipofectamine 2000. The experiment was carried out in triplicate and luminescence values normalised to control renilla luciferase. 24 hours later cells were lysed and luciferase expression detected using a dual-luciferase reporter assay system. Graph shows averages of three independent experiments. Luciferase activity was measured as raw light units (RLU) per microgram of cellular protein.

6.2.2 Back to basics- using the PGL3 basic luciferase reporter plasmid to asses TMPRSS2 promoter activity

When the newly cloned VISA plasmids were sent for sequencing it was highlighted that the original VISA plasmid already contained a *PSA* enhancer sequence. Whilst this sequence is not the entire sequence used in previous reports (~660 bp, Figure 75A), it poses questions as to what advantage/ disadvantage if any, the presence of this sequence gives to the promoter activity of Ad5-TV-CU. *In silico* analysis revealed that the PSA enhancer in VISA contains 4/6 AR binding sites of the PSA enhancer (Figure 75B), this sequence is therefore present in all VISA constructs as well as Ad5-TV-CU as the plasmid was cut at SalI and NotI and ligated into the p-shuttle vector. Therefore, we do not know what contribution this gives to the levels of transgene expression, whether this sequence is solely responsible for transgene expression, or works in combination with the *TMPRSS2* promoter to drive prostate specific expression from Ad5-TV-CU.

A



B

```
VISA            ----------------------------------AGTGTGATGG---------ATG-T 14
PSA             CTGTCCACCAGTCATGCTAGCCATACCATGATGATGATGATGATGAGAACCCCCCATGGT 240
                                                 *  .*****.         *** *

VISA            GACACAGCTCTCCGGGTGCAGGTGGTAAGCTTGGGGCTGGGGAGCCTCCCCCAGGAGCCC 74
PSA             GACACAGCTCTCCGGGTGCAGGTGGTAAGCTTGGGGCTGGGGAGCCTCCCCCAGGAGCCC 300
                ************************************************************

VISA            TATAAAACCTTCATTCCCCAGGACTCCGCCCCTGCCCTGCTGGCACCCAGAGGCTGACCA 134
PSA             TATAAAACCTTCATTCCCCAGGACTCCGCCCCTGCCCTGCTGGCACCCAGAGGCTGACCA 360
                ************************************************************

VISA            AGGCCCTCCCCATGCTGCTGGAGGCTGGACAACCCCCTCCCACACCCAGAGCTGTGGAAG 194
PSA             AGGCCCTCCCCATGCTGCTGGAGGCTGGACAACCCCCTCCCACACCCAGAGCTGTGGAAG 420
                ************************************************************

VISA            GGGAGGGAGAGCTAGCACTTGCTGTTCTGCAATTACTAGATCACCCTGGATGCACCAGGC 254
PSA             GGGAGGGAGAGCTAGCACTTGCTGTTCTGCAATTACTAGATCACCCTGGATGCACCAGGC 480
                ************************************************************

VISA            CCTGTAGCTCATGGAGACTTCATCTAGGGGACAAAGGCAGAGGAGACACGCCCAGGATGA 314
PSA             CCTGTAGCTCATGGAGACTTCATCTAGGGGACAAAGGCAGAGGAGACACGCCCAGGATGA 540
                ************************************************************

VISA            AACAGAAACAGGGGGTGGGTACGATCCCCGATTCTTCATACAAAGCCTCACGTGCCTAGA 374
PSA             AACAGAAACAGGGGGTGGGTACGATCCCCGATTCTTCATACAAAGCCTCACGTGCCTAGA 600
                ************************************************************

VISA            TCCTTTGCACTCCAAGACCCAGTGTGCCCTAAGACACCAGCACTCAGGAGATTGTGAGAC 434
PSA             TCCTTTGCACTCCAAGACCCAGTGTGCCCTAAGACACCAGCACTCAGGAGATTGTGAGAC 660
                ************************************************************

VISA            TCCCTGATCCCTGCACCACTCTGAGACCAGAAACTAGAACTTTTATTCCTCATGCTCCTG 494
PSA             TCCCTGATCCCTGCACCACTCTGAGACCAGAAACTAGAACTTTTATTCCTCATGCTCCTG 720
                ************************************************************

VISA            AAATAGATGTCTTGGCATTTAGTACATTCTTTTCCTTGCACTCCCAACCCAGAATCCAGC 554
PSA             AAATAGATGTCTTGGCATTTAGTACATTCTTTTCCTTGCACTCCCAACCCAGAATCCAGC 780
                ************************************************************

VISA            TCCACAGATACATTGCTACTGTCATCATAAAAAGATCTTGTGGTCCACAGATCCTCTAGC 614
PSA             TCCACAGATACATTGCTACTGTCATCATAAAAAGATCTTGTGGTCCACAGATCCTCTAGC 840
                ************************************************************

VISA            CCAGAAATATGAGTCTCCCAAAGTTCCCTAGCATTTCAAAATCCAACGTGCAGCAAACAA 674
PSA             CCAGAAATATGAGTCTCCCAAAGTTCCCTAGCATTTCAAAATCCAACGTGCAGCAAACAA 900
                ************************************************************

VISA            TGTACTAGTGG
PSA             TGTACTAGTCG
                ********* *
```

Figure 75 *In silico* analysis of PSA enhancer sequence already existing in the original C-VISA vector (Prof Hung).

A Plasmid map highlighting the PSA enhancer upstream of the CCKAR promoter in the original C-VISA promoter. **B** DNA sequence alignment of 660 bp of the PSA enhancer identified in C-VISA (top line) alongside the wild type PSA sequence (bottom line), containing 4/6 published AR binding sites. *=conserved sequence

The presence of the PSA enhancer in the VISA plasmid posed questions as to the nature of the promoters in terms of their prostate specific activity. In order to establish the true activity of the L-ARE, W-ARE and W-enhancer the three regions containing AREs were cloned into the PGL3 basic empty vector (Promega) and named L-PGL3, W-PGL3 and WE-PGL3 (W enhancer). For full details of location, size, sequencing results and number of AREs see appendix section 8.1.

6.2.2.1 The previously identified TMPRRS2 AREs do not drive prostate specific expression in a PGL3-B reporter vector

L-PGL3, W-PGL3 or WE-PGL3 were transfected into AR-positive 22RV1 cells, AR-negative HEK293 (normal human embryonic kidney cells) and Panc1 (Pancreatic cancer cell line) cells. Surprisingly, none of the regions were able to drive prostate specific expression (Figure 76). The L-PGL3 plasmid generated similar levels of luciferase expression in both 22RV1 AR-positive PCa cells and Panc1 pancreatic cancer cells, with lower expression in HEK293. Similarly, when the W-PGL3 plasmid was transfected into all three cell lines there were similar levels of luciferase expression in 22RV1 and Panc1 cells, but the levels of luciferase expression in 22RV1 were 4.1-fold higher than in HEK293 and 15-fold higher than the PGL3-B vector, demonstrating strong promoter activity in comparison to L-PGL3.

The previously published TMPRSS2 enhancer, located -13.5 kb upstream of Exon1 of *TMPRSS2* (Wang et al. 2007), demonstrated negligible activity, similar to PGL3-B vector.

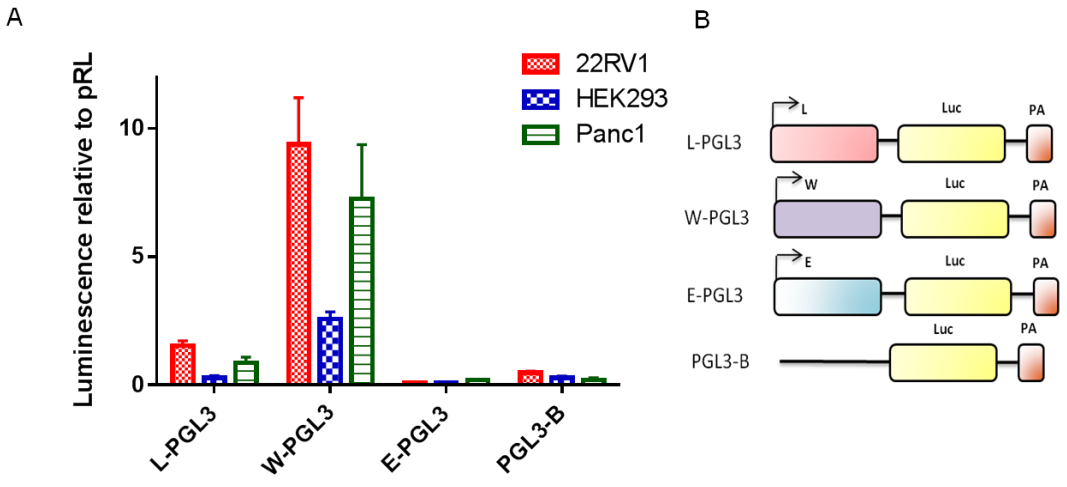


Figure 76 E-ARE, L-ARE and W-ARE are not prostate specific promoter regions alone

A. Cells were co-transfected with 150 ng/well of L-PGL3, W-PGL3 WE-PGL3 or PGL3-B and 15 ng/well renilla luciferase under the control of the SV40 promoter (pRL-SV40). Cells were lysed the following day and luciferase expression detected using a dual luciferase reporter assay system. Luciferase expression is relative to the control pRL. Data is a mean of 3 independent experiments. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein. **B**. Diagram of promoter constructs used to express the luciferase reporter gene.

6.2.2.2 Combination of the L and W AREs restores prostate specific luciferase transgene expression to AR-positive 22RV1 cells

Combining the L-ARE with the W-ARE to make LW-PGL3 resulted in 8.9 and 1.5-fold higher luciferase expression in 22RV1 cells transfected with LW-PGL3 in comparison to L-PGL3 and W-PGL3, respectively (Figure 77). However, as well as increasing expression in 22RV1 cells, Panc1 and HEK293 cells transfected with LW-PGL3 also demonstrate increased promoter activity in comparison to L-PGL3. In HEK293 cells this promoter activity was the same as seen after transfection with W-PGL3 alone, however the activity in Panc1 cells was 2-fold lower than when W-PGL3 alone was transfected alone. This suggests that a combination of the L and W AREs results in higher levels of more prostate specific expression than L or W alone.

The combination of the TMPRSS2 enhancer (E) with L-PGL3 (EL-PGL3) drove 5-fold higher luciferase expression than E-PGL3 alone in 22RV1 cells, but more importantly, increased luciferase expression 1.4-fold in comparison to L-PGL3. The combination of E and L has no effect on luciferase expression in either of the AR-negative Panc1 or HEK293 cells, demonstrating possible low level enhancer activity.

Furthermore, when the TMPRSS2 enhancer was cloned in combination with W-PGL3 (EW-PGL3), luciferase expression increased 1.2-fold in 22RV1 cells in comparison to cells transfected with W-PGL3 alone. However, EW-PGL3 was also marginally more active in Panc1 cells, showing that the combination of E and W does not have the same effect on restricting tissue type specific expression as the combination of L and W AREs.

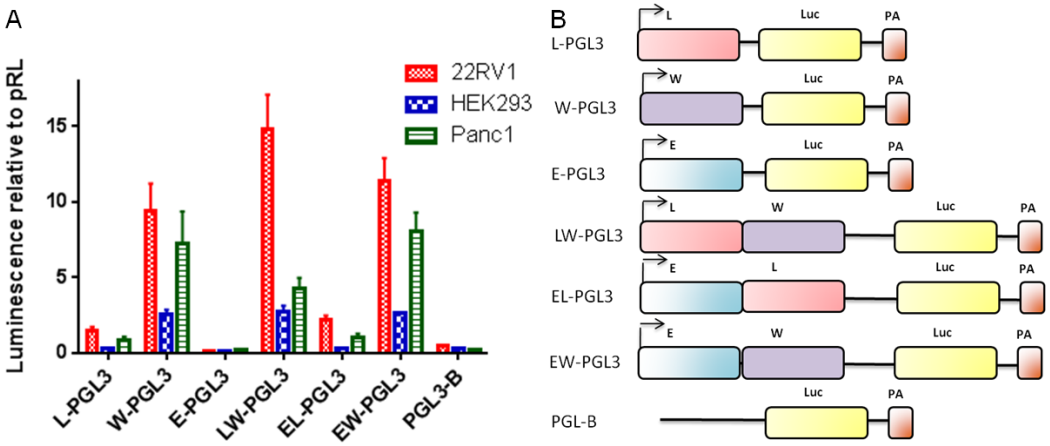


Figure 77 Combination of the L-ARE and W-ARE restores prostate specific expression to 22RV1 cells

A. 2x10^4 22RV1, HEK293 or Panc1 Cells were co-transfected with 150 ng/well of the luciferase plasmids shown and 15 ng/well renilla luciferase under the control of the SV40 promoter (pRL-SV40) using lipofectamine 2000. Cells were lysed the following day and luciferase expression detected using a dual luciferase reporter assay system. Data is a mean of four independent experiments. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein. **B**. Diagram of promoter constructs used to express the luciferase reporter gene.

In order to further show that the TMPRSS2 promoter elements are superior to PSA promoter or enhancer elements, in an empty vector, the PSA promoter and chimeric PSA promoter/enhancer were cloned into PGL3-B (Figure 78) and activity compared to the *TMPRSS2* promoter constructs. Surprisingly, when Panc1 cells were transfected with P-PGL3, containing the PSA promoter alone, higher levels of luciferase transgene expression were detected than in 22RV1 cells transfected with the same plasmid (Figure 78). Additionally, luciferase expression levels in P-PGL3 transfected Panc1 were 1.8-fold higher in comparison L-PGL3 transfected Panc1. This expression was reduced when Panc1 cells were transfected with PEP-PGL3, containing the PSA promoter and enhancer, indicating an inhibitor effect of the PSA enhancer on PSA promoter driven expression in these cells.

Luciferase expression levels in 22RV1 cells transfected with P-PGL3 were 1.3-fold higher than cells transfected with L-PGL3, suggesting that the PSA promoter drives higher levels of luciferase transgene expression in 22RV1 cells than the L promoter of TMPRSS2. However, W-PGL3 remains a much stronger promoter, driving 4.4-fold higher levels of luciferase expression than P-PGL3 in 22RV1 cells. When the PSA enhancer is added in combination with the PSA promoter there is reduced luciferase expression in 22RV1 cells, suggesting an inhibitory effect of this combination.

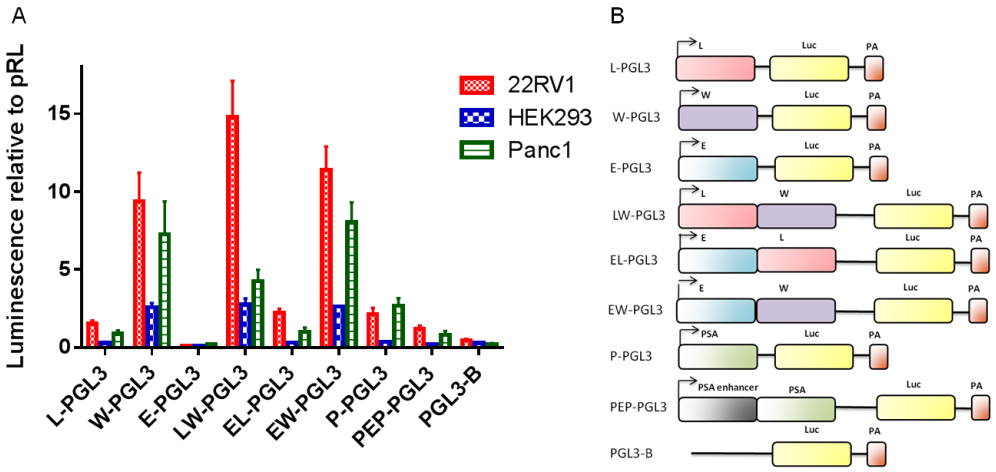


Figure 78 Combination of AREs from TMPRSS2 and PSA and evaluation of activity

A. 2x10^4 22RV1, HEK293 or Panc1 cells were co-transfected with 150 ng/well of the luciferase plasmids shown, in combination with 15 ng/well renilla luciferase under the control of the SV40 promoter (pRL-SV40) using lipofectamine 2000. 24 h later cells were lysed and luciferase expression detected using a dual-luciferase reporter assay system. Average results from four independent experiments are shown. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein. **B**. Diagram of promoter constructs used to express the luciferase reporter gene.

6.2.2.3 Combination of two L AREs with W looses AR-positive cell line specificity.

A previous publication using the PSA promoter stated that a combination of the PSA promoter with two PSA enhancers drove high and prostate specific expression (Latham et al. 2000). To investigate whether this is also the case with the TMPRSS2 promoter, I cloned two L-AREs upstream of W-ARE in the PGL3-B plasmid, as well as a number of other plasmids containing the PSA enhancer in combination with L-ARE and W-ARE (Figure 79B). Luciferase expression in 22RV1 cells transfected with LLW-PGL3 was lower in comparison to LW-PGL3, but when Panc1 cells were transfected with LLW-PGL3 expression increased 1.6-fold in comparison to cells transfected with LW-PGL3. Levels of luciferase expression in HEK293 cells were similar whether transfected with LW-PGL3 or LLW-PGL3 (Figure 79A).

To establish whether the high levels of specific expression in the L-VISA plasmid are due to the interaction between the PSA enhancer present in the VISA plasmid and the L-ARE, the PSA enhancer was cloned in combination with either the L or W-ARE (PW-PGL3, PL-PGL3). There was no difference in luciferase transgene expression levels when the PSA enhancer was cloned in combination with either the W-ARE or the L-ARE in comparison to the chimeric PSA promoter/enhancer,

except that the prostate specificity appeared to increase when the PSA enhancer was cloned in combination with L. (Figure 79A). This was shown by reduced expression in Panc1 cells, similar to what is seen in L-VISA. When luciferase transgene expression is compared between W-PGL3 and L-GPL3 alone and in combination with the PSA enhancer (PW-PGL3 or LW-PGL3) in 22RV1 cells there is a 3.1 and 1.3-fold reduction in luciferase transgene expression respectively. There is also decreased expression in HEK293 and Panc1 cells transfected with PW-PGL3 or PL-PGL3 in comparison to W-PGL3 and L-PGL3. This mirrors the results seen when the PSA enhancer was combined with the PSA promoter, showing an inhibitory effect of the PSA enhancer in combination with prostate tissue specific promoters.

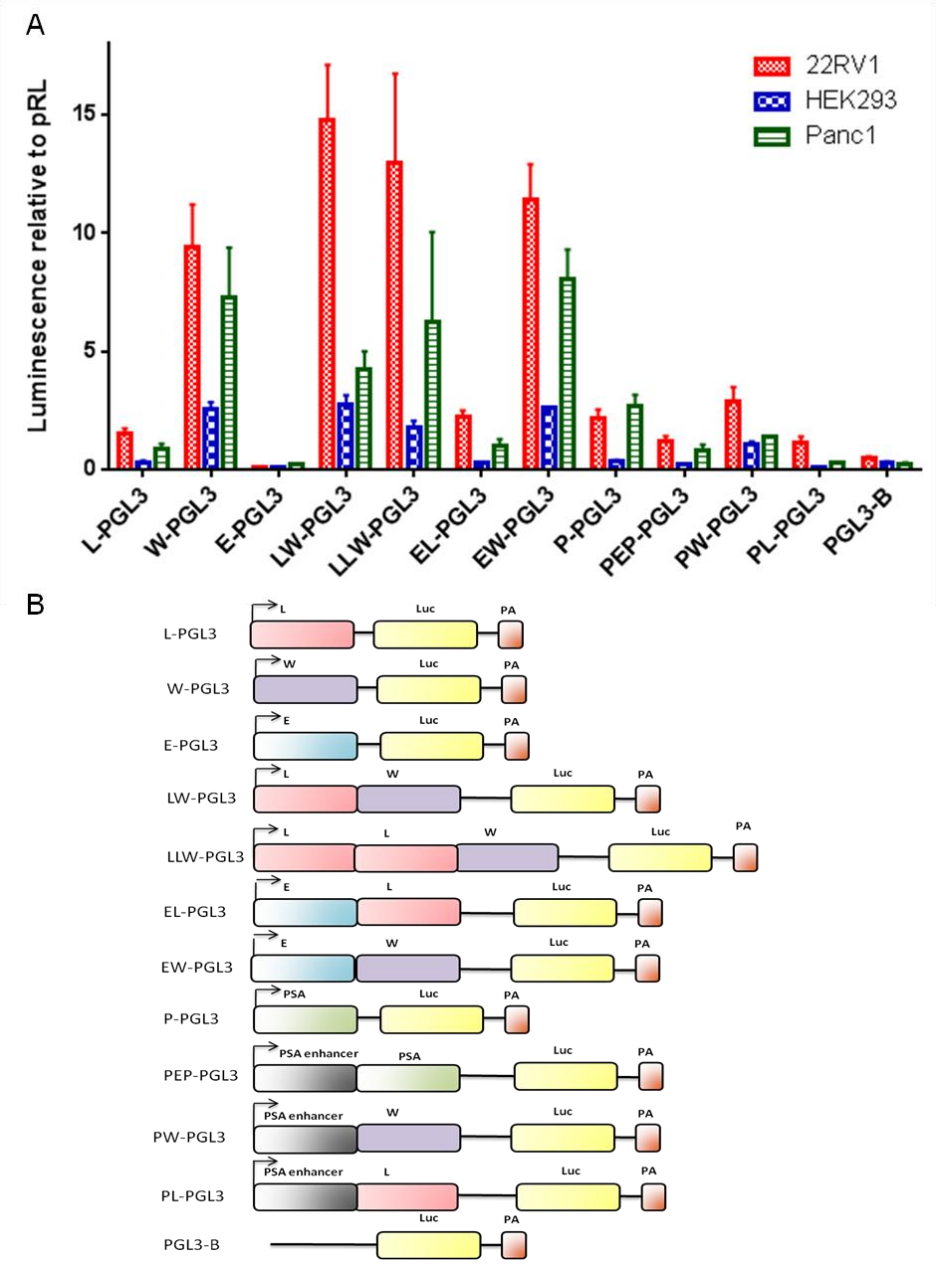


Figure 79 Combination of PSA enhancer with W and L inhibits promoter activity compared to L and W alone

A. 2x10^4 22RV1, HEK293 or Panc1 cells were co-transfected with 150 ng/well of the luciferase plasmids shown in combination with 15 ng/well renilla luciferase under the control of the SV40 promoter (pRL-SV40) using lipofectamine 2000. 24 hours later cells were lysed and luciferase expression detected using a dual-luciferase reporter assay system. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein. Results are a mean of four independent experiments. **B**. Diagram of promoter constructs used to express the luciferase reporter gene.

6.2.2.4 Comparison of the new optimal TMPRSS2 promoter, LW, with constitutively active SV40 and CMV promoters.

After demonstrating that the LW-promoter was able to drive the highest levels of prostate specific expression, I investigated whether the levels of luciferase expression after transfection were comparable to the constitutively active SV40 and CMV promoters. SV40-PGL3 (Promega) was transfected together with L-PGL3, W-PGL3 and LW-PGL3 into HEK293, Panc1, LNCaP-104-S and 22RV1 cells and luciferase expression assessed 24 h later. Interestingly, SV40-PGL3 expression remained less than 10-fold higher than PGL3-B in all cell lines except for Panc1, which demonstrated more than 20-fold higher luciferase expression than PGL3-B and up to 10-fold higher luciferase expression than in 22RV1 cells, this was unexpected from a universal promoter (Figure 80). As a consequence of this, an alternative SV40-PGL3 plasmid was obtained from a different source (SV40-PGL3-2, Kunal Shah, Molecular Oncology, Barts Cancer Institute), but showed a similar pattern of expression across all four cell lines (data not shown). Despite this, LW-PGL3 drove 4.5-fold higher luciferase expression in comparison to SV40-PGL3 in 22RV1 cells, but remained 3.6-fold, 4.7-fold and 6.8-fold weaker than SV40-PGL3 in LNCaP-104-S, HEK93 and Panc1 respectively.

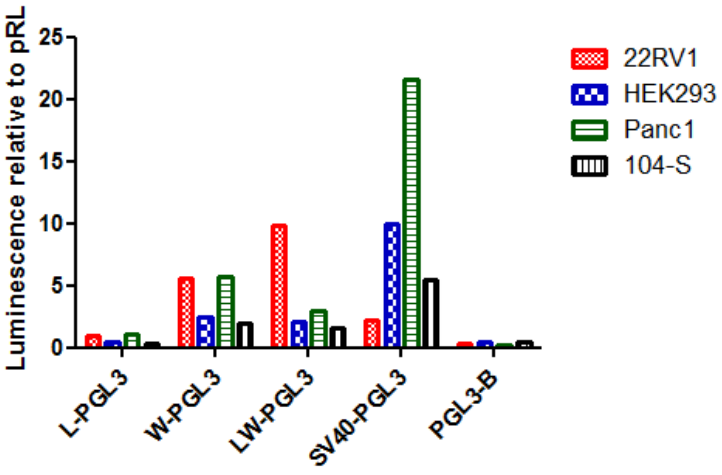


Figure 80 LW-PGL3 drives 4.5-fold higher levels of luciferase than the constitutively active SV40-PGL3 vector

$2x10^4$ 22RV1, HEK293 and $1x10^4$ Panc1 and LNCaP-104-S cells were co-transfected with 150 ng/well of the luciferase plasmids shown, in combination with 15 ng/well renilla luciferase under the control of the SV40 promoter (pRL-SV40) using lipofectamine 2000. 24 h later cells were lysed and luciferase expression detected using a dual-luciferase reporter assay system. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein.

I later cloned the CMV promoter into the PGL3-B vector and compared the levels of luciferase activity once again between L-PGL3, W-PGL3, LW-PGL3, and CMV-PGL3 transfected 22RV1 cells. CMV-PGL3 drove 214-fold higher luciferase expression than LW-PGL3, demonstrating that the LW promoter alone in the basic vector is not capable of driving higher levels than the constitutively active CMV promoter (Figure 81).

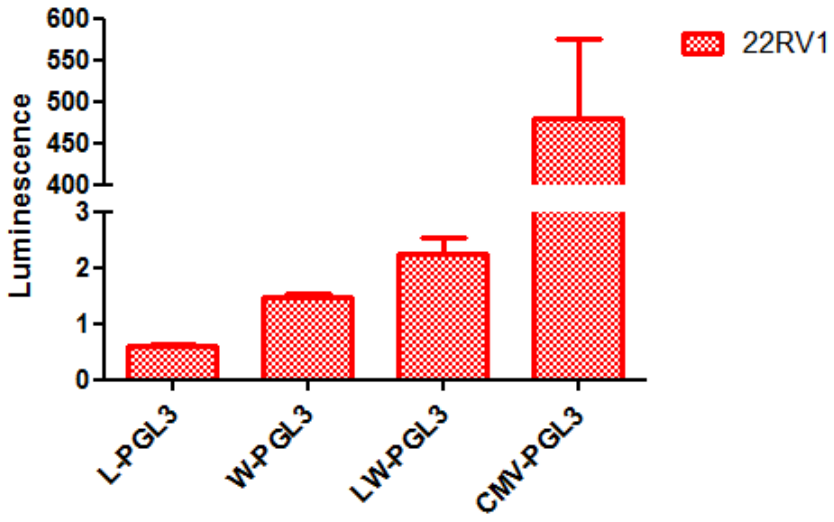


Figure 81 CMV promoter drives 214-fold higher levels of luciferase expression than the optimal LW-promoter

$2x10^4$ 22RV1 cells were co-transfected with 150 ng/well of the luciferase plasmids shown in combination with 15 ng/well renilla luciferase under the control of the SV40 promoter (pRL-SV40) using lipofectamine 2000. 24 h later cells were lysed and luciferase expression detected using a dual-luciferase reporter assay system. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein.

6.2.2.5 LW-PGL3 is active in AR-positive BCa cell line MM453

After a dose response to Ad5-TV-CU alone was generated in the AR-positive BCa cell line MM453, it was desirable to evaluate luciferase transgene expression from the new TMPRSS2 reporter plasmid in this cell line, as well as MCF7 AR-positive cells, to establish whether future studies should include AR-positive BCa cell lines. MCF7 cells express lower levels of AR than MM453 cells, and therefore, unsurprisingly have the lowest luciferase transgene expression levels after transfection with all plasmids, except for LLW-PGL3 (Figure 82). Luciferase transgene expression from LW-PGL3 transfected MM453 cells was 5.1-fold higher than L-PGL3, but 1.2-fold lower than W-PGL3, showing that the combination of L

and W does not have the same enhancer effect in MM453 cells as it does in 22RV1 cells. There was very little difference in the level of luciferase transgene expression in LW-PGL3 transfected MCF7 cells in comparison to L-PGL3 and W-PGL3. Furthermore, luciferase transgene expression from L-PGL3 transfected MM453 and MCF7 cells was 1.3-fold and 1.6-fold lower than in 22RV1 cells respectively and W-PGL3 luciferase expression in MM453 and MCF7 cells was 7.4-fold and 1.9-fold lower than 22RV1 cells respectively.

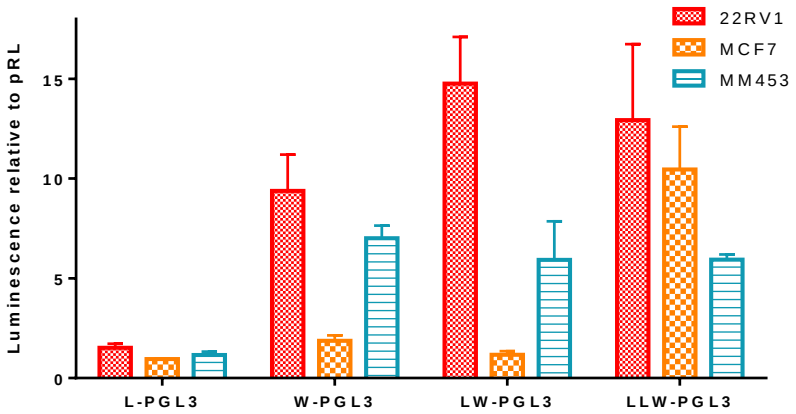


Figure 82 Luciferase transgene expression is higher in MM453 AR-positive cells transfected with TMPRSS2 luciferase plasmids compared to MCF7

$2x10^4$ 22RV1, MCF7 or MM453 cells were transfected with 150 ng/well L-PGL3, W-PGL3, LW-PGL3 or LLW-PGL3 in combination with 15 ng/well renilla luciferase under the control of the SV40 promoter (pRL-SV40) using lipofectamine 2000. 24 h later cells were lysed and luciferase expression detected using a dual-luciferase reporter assay system. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein.

6.2.3 Stimulation/inhibition of AREs in TMPRSS2 constructs by mibolerone, Bicalutamide and Estradiol.

It was important to establish whether transgene expression from the plasmids containing the various TMPRSS2 promoters both alone, or in combination could be increased upon addition of the synthetic androgen mibolerone or decreased upon addition of the anti-androgen Bicalutamide. I was previously unable to detect increased/decreased CD/UPRT transgene expression by western blot in both LNCaP and 22RV1 cells after treatment with mibolerone, Bicalutamide or Estradiol (see sections 4.2.1.3 and 4.2.4.5). Therefore, the same experiments were repeated using luciferase assays, as they are more sensitive to slight increases or decreases in transgene expression.

The AR responsive reporter plasmid MMTV-Luc, containing an inducible hormone response element regulated by androgen and glucocorticoid, was included in all

experiments as a control for drug activity. There was no difference in luciferase expression levels when any of the plasmids were combined with either mibolerone or Bicalutamide in 22RV1 cells (Figure 83). However, 10-fold more luciferase expression was detected when 22RV1 cells transfected with MMTV-Luc were treated with either mibolerone or Estradiol, confirming that both drugs are active and capable of stimulating AR. Interestingly, there was 20% more expression when LW-PGL3 was treated with Estradiol, however this was not comparable to the 10-fold increase in expression when the MMTV-Luc plasmid was transfected in combination with Estradiol. This experiment was repeated three times and expression consistently increased when LW-PGL3 was transfected in combination with Estradiol, however the difference was not significant p=>0.05.

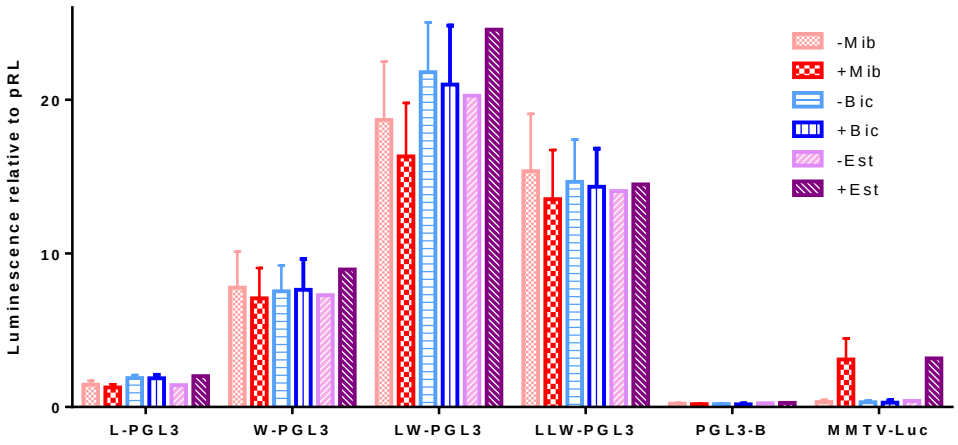


Figure 83 Luciferase transgene expression increases by 20% when LW-PGL3 is transfected into 22RV1 cells in combination with Estradiol

$2X10^4$ 22RV1 cells were seeded in 10% C/S media and transfected the following day with L-PGL3, W-PGL3, LW-PGL3, LLW-PGL3, PGL3-B or MMTV-LUC. 4 h after transfection media was replaced with media containing 1 nM mibolerone, 5 µM Bicaluatamide or 10 nM Estradiol. 24 h later cells were lysed and luciferase expression detected using a dual-luciferase reporter assay system. Luciferase activity was measured as raw light units (RLU) per microgram cellular protein (n=3). p=>0.05

6.3 DISCUSSION

Previous studies have suggested that gene specific promoters are capable of driving low levels of expression; however they are often not tissue specific (Riegman et al. 1991) as tissue specificity is most often a result of the gene specific enhancers found upstream or downstream of the tissue specific promoter (Pang et al. 1997).

PSA is the most extensively studied prostate specific promoter and has been widely used for tissue specific restriction of transgene expression in PCa therapy (Wu, Matherly, et al. 2001, Li et al. 2005, Lee et al. 2002, Latham et al. 2000). However, studies using the PSA promoter have ultimately failed due to poor levels of transgene expression from tissue specific promoters. Efforts to increase transgene expression from these promoters by producing chimeric promoters/enhancers drove up to 1000-fold higher expression in comparison to the PSA promoter alone (Latham et al. 2000). Further studies that have included the fusion of the regulatory regions of both the PSA and PSMA genes that were able to drive high levels of luciferase transgene expression in both AR expressing and non AR expressing cell lines, providing potentially promising therapies for the treatment of CRPC (Lee et al. 2002). This chimeric enhancer was subsequently inserted into an adenoviral vector and demonstrated dramatic inhibition of CWR22rv tumours *in vivo* (Li et al. 2005). Despite promising *in vitro* and *in vivo* studies, these systems have never entered clinical trials due to poor efficacy. The *TMPRSS2* promoter has never before been investigated as a prostate specific promoter; I therefore initially wanted to compare transgene expression levels from the *TMPRSS2* promoter sequences in L-VISA and W-VISA to the previously validated PSA promoter, by replacing the *TMPRSS2* promoter in L-VISA with the PSA promoter region and the stronger chimeric PSA promoter/enhancer region. L-VISA and W-VISA drove higher levels of transgene expression in 22RV1 in comparison to P-VISA, suggesting that the *TMPRSS2* promoter is superior to *PSA* in this construct. Additionally, despite previous *in vitro* studies with Ad5-TV-CU (containing the L-ARE), that demonstrated AR-positive cell line specific transgene expression after transfection and infection, I wanted to go back to basics in order to establish the promoter activity of the two plasmids without the interference of the PSA enhancer and cloned both the L-ARE and W-ARE into the PGL3-B

vector, as well as the PSA promoter alone or in combination with the enhancer (producing L-PGL3, W-PGL3, P-PGL3 and PWE-PGL3).

I concluded that both L-VISA and W-VISA drove higher levels of luciferase transgene expression in 22RV1 cells than the P-VISA. Additionally, comparison of the new PGL3-B vectors containing the L and W-AREs confirmed higher activity from the *TMPRSS2* promoter elements than the PSA promoter alone or in combination with its enhancer. Interestingly, similar levels of luciferase transgene expression were detected in 22RV1 AR-positive and Panc1 AR-negative cell lines after transfection with both L-PGL3 and W-PGL3, rendering the promoter unspecific. This lack of prostate specific expression could be due to the lack of other transcription factors required in addition to AR, which are responsible for the transcriptional activation of the TMPRSS2 promoter. Alternatively, Panc1 cells may express AR, or transcriptional regulators of *TMPRSS2* that are as of yet unknown, as low level expression of TMPRSS2 RNA has previously been described in the pancreas (Lin et al. 1999). I also detected very low level luciferase expression in the normal human embryonic kidney cell line (HEK293) and TMPRSS2 RNA expression has also been detected in the kidney at low levels (Lin et al. 1999), further confirming the possible unspecific nature of the TMPRRS2 regulatory regions.

I later found that the VISA expression cassette contained 660 bp of the PSA enhancer DNA that I was previously unaware of. This enhancer sequence, consisting of 4/6 of the previously published AREs of the PSA enhancer, was clearly able to drive some low level transgene expression alone, demonstrated by the high background luciferase expression in 22RV1 cells transfected with E-VISA (promoter-less empty-VISA). Surprisingly, luciferase expression from P-VISA did not seem to be restricted to AR-positive cell lines, this is possibly due to the interaction between the 660 bp enhancer present in VISA and the PSA promoter. The promoter may require all 6 AREs of the PSA enhancer, allowing for a more complete set of transcription factors implicated in endogenous gene regulation to drive prostate specific expression from the PSA promoter. Consequently, the presence of 600 bp of the PSA enhancer may affect transgene expression from this plasmid. A similar pattern was seen in the PGL3 plasmids that did not contain the VISA sequence. The PSA promoter alone did not drive specific expression in AR-positive cell lines, however a combination with the PSA enhancer resulted in decreased expression in both 22RV1 and Panc1 cells, with an overall effect of

increasing specific expression in 22RV1 cells in comparison to Panc1 (Figure 79A).

Firstly, whilst both the L and W AREs have failed to demonstrate clear prostate specific expression in the 22RV1 cell line, the L-ARE, present in L-VISA is capable of driving high levels of prostate specific transgene expression in Ad5-TV-CU. This lead to questions as to what contribution the PSA enhancer, that was already present in VISA was giving towards the activity and tissue specificity in L-VISA and as a consequence Ad5-TV-CU and whether this could be replicated using TMPRSS2 enhancer regions. To investigate this further, I aimed to increase prostate specific transgene expression by a combining the TMPRSS2 enhancer (WE) with either L-PGL3 or W-PGL3. Unfortunately this combination did not enhance the promoter activity of these plasmids. There could be two potential reasons for the poor enhancer activity of the combination with the TMPRSS2 enhancer: 1) The TMPRSS2 enhancer requires long-distance interaction with a promoter though chromatin looping in order to drive transcription of TMPRSS2, as previously described (Wang et al. 2007) and therefore when cloned in direct sequence, loses its interaction with *TMPRSS2* promoter regions. 2) The previously identified TMPRSS2 enhancer does not in fact have enhancer activity, contrary to previous reports suggesting it interacts with the TMPRSS2 promoter to drive higher levels of prostate specific expression (Wang, Carroll, and Brown 2005).

After failing to increase transgene expression by combining the previously published TMPRSS2 upstream enhancer, I cloned, in sequence, the L-ARE that drives prostate specific expression in VISA, with W-ARE, that drives high levels of unspecific luciferase transgene expression in all cell lines, resulting in increased prostate specific transgene expression after LW-PGL3 transfection. Evidence suggests that the L-ARE and W-ARE work in combination to drive high levels of prostate specific expression. This is similar to the previous studies with the PSA promoter and enhancer elements, which have demonstrated that chimeric promoters, comprised of a tissue specific promoter in combination with an enhancer, drive high levels of tissue specific expression in prostate cells (Pang et al. 1997, Latham et al. 2000). In fact, the DNA sequence upstream of the PSA gene promoter was found to perform not only as an enhancer but also as a promoter (Pang et al. 1997). We therefore propose a model, whereby the L-ARE acts as the promoter of TMPRSS2 and through interaction with the W-ARE,

functioning as both a promoter and enhancer, drives high levels of prostate specific transgene expression.

Despite the higher levels of transgene expression in 22RV1 AR-positive cell lines in comparison to AR-negative cell lines transfected with LW-PGL3, there is still some expression of LW-PGL3 in Panc1 cells. Previous studies with the PSA promoter have shown that promoter activation does not solely depend on activation by AR, but is also dependent on other promoter-DNA-binding proteins produced exclusively in prostate cells (Pang et al. 1995). The full repertoire of prostate specific transcription factors has not yet been fully elucidated, and could vary from promoter to promoter. It is possible that the LW *TMPRSS2* promoter is activated by a number of transcription factors exclusive to the prostate, or that these factors are also expressed in cell lines where TMPRSS2 expression has been previously detected, for example the pancreas (Lin et al. 1999), explaining activity of LW-PGL3 in Panc1 cells. Further investigation into this is necessary, through screening a larger number of AR-positive and negative cell lines.

After establishing that a combination of the L and W regions of TMPRSS2 drove the highest levels of transgene expression, I wanted to compare the activity of this new optimal promoter with the constitutively active SV40 and CMV promoters. SV40-PGL3 expression was 4-fold and 10-fold lower in 22RV1 and LNCaP-104S cells respectively, in comparison to Panc1, suggesting that SV40 does not function well as a constitutive promoter in LNCaP-104-S and 22RV1 cells. In fact, other labs have experienced similar difficulties in establishing high levels of luciferase expression from the SV40 promoter in combination with its enhancer in LNCaP cells, where they found that the addition of the SV40 promoter did not increase luciferase transgene expression. Contrarily in PC-3 cells there was a huge increase in reporter activity when the SV40 promoter was combined with its enhancer, suggesting that SV40 promoter activity is potentially repressed in some prostate cell lines (Chung, Isaacs, and Simons 2007). SV40 has also been shown to drive 3-fold lower levels of expression in LNCaP and MDA PCa 2b cells in comparison to HeLa and NMU cells (Logg et al. 2002). This lead to investigating whether a second constitutively active promoter, CMV, would behave differently in 22RV1 cells. Luciferase expression from the CMV promoter was ~214-fold higher than the LW promoter in 22RV1 cells and considerably higher than the previous SV40 promoter. Although the LW promoter alone cannot drive similar levels of luciferase expression to the constitutively active CMV promoter, nor the L-VISA

TSTA system that is currently in Ad5-TV-CU. Inclusion of the LW promoter in the VISA expression cassette will potentially drive levels of luciferase expression that are equal to, or greater than the CMV promoter. LW-PGL3 is able to drive 8.9-fold and 1.5-fold higher expression than both the L-PGL3 and W-PGL3 promoters respectively (section 6.2.2.2). These promoters are currently present in L-VISA and W-VISA and already drive similar levels of luciferase expression to the CMV promoter. Thus, increasing both the prostate specificity and activity by incorporation of the LW promoter into the VISA TSTA could increase the therapeutic efficacy of a new adenovirus construct.

After discovering promising results in 22RV1 AR-positive cells with the new LW-PGL3 chimeric promoter, I wanted to establish whether the same activity could be achieved in MM453 and MCF7 AR-positive BCa cell lines. LW-PGL3 is active in MM453 cells, but luciferase transgene expression levels do not increase in this cell line in comparison to W-PGL3 alone, contradictory to what we see in 22RV1 cells. The difference in enhancer effect of LW-PGL3 between MM453 cells and 22RV1 cells is possibly due to: 1) lower levels of AR expression in MM453 compared to 22RV1 or 2) absence of prostate specific transcription factors required to further activate the LW chimeric *TMPRSS2* promoter. Luciferase transgene expression from LW-PGL3 in MCF7 cells is 1.3-fold higher than L-PGL3 and 1.6-fold lower than W-PGL3. This low level transgene expression after transfection in MCF7 cells is most probably due to very low levels of AR expression in this cell line in comparison to 22RV1 and MM453 (Cochrane et al. 2014). Thus, luciferase transgene expression levels in MM453 cells transfected with LW-PGL3 suggests that the new virus with the optimal LW promoter could be effective against some AR-positive BCas that currently have very limited therapeutic interventions.

It was also important to establish the androgen responsiveness of the L and W AREs both alone and in combination, I therefore employed luciferase assays plus and minus mibolerone or Bicalutamide in combination with the different plasmids in 22RV1 cells. Furthermore, previous studies have suggested that the *TMPRSS2* promoter is under the transcriptional control of ER (Setlur et al. 2008, Bonkhoff and Berges 2009). I therefore also employed the ER-α agonist, Estradiol, to see if it would stimulate expression from the TMPRSS2 promoter plasmids. I saw a 10-fold increase in luciferase transgene expression when MMTV-Luc was treated with both Mibolerone and Estradiol, demonstrating the activity of both drugs. The

MMTV-Luc plasmid is a control for AR activity and should be stimulated by mibolerone, a synthetic AR agonist, however the increase in luciferase expression from MMTV-Luc treated with Estradiol highlights the cross-reactivity between the ER and AR pathways. I also saw a small increase in luciferase expression when LW-PGL3 was treated with Estradiol, however this was not anywhere near the 10-fold induction of MMTV-Luc.

It is most probable that we do not see a difference in expression levels after treatment in androgen-independent 22RV1 cells due to the mutated AR in this cell line (previously discussed in section 4.3). The same experiments would need to be repeated in androgen-dependent cell lines LNCaP-104-S, LNCaP-CDXR3 or LNCaP-104R1 to truly establish the AR-responsiveness of these plasmids. Preliminary studies in LNCaP-CDXR3 cells have demonstrated much lower levels of luciferase expression from all plasmids, most probably due to the poor transfectability of these cell lines (discussed earlier). Future work would need to include transfection by electroporation in this cell line to increase transfection efficiencies.

In conclusion, the combination of L and W AREs restore prostate specificity of the W promoter, by simultaneously increasing transgene expression in 22RV1 cells, whilst decreasing expression in Panc1. LW is therefore a more superior promoter to L, which is currently in Ad5-TV-CU, and could be used to drive higher and more specific transgene expression for adenoviral gene therapy in PCa, with potential clinical applications for the treatment of AR-positive BCa. If incorporated into the VISA vector, the LW promoter is capable of driving similar or potentially even higher levels of luciferase expression than the constitutively active CMV promoter. However, despite demonstrating prostate specificity, there is no increase in luciferase expression from any of the plasmids when treated with mibolerone, and only a slight increase when treated with Estradiol. Further investigation into what causes prostate specific activation of these promoter elements may help to fully elucidate the role of TMPRRSS2 in PCa progression and provide potential targets for future therapies.

I also wanted to investigate the effect of the PSA enhancer in VISA on activity of non-prostate specific promoters. N-VISA contains the promoter element of the gene NKAIN2, this gene is not prostate specific, however 2-fold higher luciferase expression was detected in 22RV1 AR-positive cells after transfection with N-VISA

in comparison to any other cell line. A previous study has shown that the PSA enhancer can work together with other prostate specific and non-prostate specific promoters to drive high levels of prostate specific expression (Lee et al. 2002, Chapel-Fernandes et al. 2006), where fusion of the PSA enhancer to the constitutively active PGK promoter increased transgene expression specifically in LNCaP cells. Furthermore, C-VISA was used as a control for luciferase expression in Panc1 cells. However, this plasmid showed no activity in Panc1 cells, and was in fact more active in 22RV1 cells, despite previous publications showing this promoter was capable of driving high levels of transgene expression in Panc1 (Xie et al. 2007). The PSA enhancer present could possibly be responsible for this high expression in 22RV1, increasing transgene expression from a non-prostate promoter. Finally, C-VISA that Xie et al gifted us may be different to the one used in previous studies, possibly explaining the lack of activity from this promoter in Panc1.

CHAPTER SEVEN

FINAL DISCUSSION AND FUTURE DIRECTIONS

7.1 Implications of the findings

The rationale behind this research was as a proof of concept to create a non-replicating adenovirus that was capable of infecting and killing PCa cells specifically, by taking advantage of the androgen regulated expression of the *CD/UPRT* suicide gene, due to the TMPRSS2 promoter. Once proven, the ultimate goal was to engineer an oncolytic virus that is replication selective and expresses CD/UPRT from the optimal TMPRSS2 chimeric promoter. This will be produced by replacing the E1B or E3 genes with the optimal construct, resulting in the death of cancer cells, both through viral gene replication, and through tissue specific expression of the CD/UPRT transgene.

Watchful waiting is employed as the current mainstay treatment for localised prostate cancer; however as it progresses to locally advanced cancer, depending on the age and stage of the patient, hormonal therapies or more aggressive treatment may be given. Aggressive treatments usually incur lifelong unwanted side effects and prolonged treatment with hormonal therapies such as the antiandrogen Bicalutamide can lead to castration resistant PCa; therefore it is necessary to find a less invasive, yet effective treatment for PCa. Enzyme prodrug therapy has been widely investigated for the treatment of many cancers, including prostate cancer. Most often expression of the enzyme is controlled by a tissue specific promoter, restricting expression to the cell type of choice, and therefore limiting off-target toxicity. Previous studies utilizing tissue specific promoters for this purpose have not yet made phase III clinical trials. In this study we aimed to produce a highly efficacious and targeted adenovirus for prostate cancer, which would have potentially massive clinical implications for the treatment of early and late stage PCa.

The data presented in this thesis demonstrated that a non-replicating adenovirus, with CD/UPRT gene expression controlled by androgen responsive elements of the *TMPRSS2* gene, enhances cell death in combination with 5-FC in AR-positive prostate cancer cell lines, 22RV1, LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 *in vitro,* while having no significant toxicity in AR-negative cell lines, including normal immortalized prostate cells (PNT1A), primary prostate epithelial cells (PrEC) and the metastatic prostate cancer cell line PC3.

Since fusion of the regulatory region of *TMPRSS2* to the *ERG* oncogene is prevalent in >50% of prostate cancers, we believed the *TMRPSS2* promoter region would drive high levels of prostate specific expression. I demonstrated the increased activity of the *TMPRSS2* promoter regions in comparison to the chimeric *PSA* promoter and enhancer. I also generated a new chimeric *TMPRSS2* promoter composed of a combination of one *TMPRSS2* region upstream of Exon1 with another *TMPRSS2* region upstream of Exon2. This new chimeric promoter (LW) can drive 8.9-fold higher levels of transgene expression that the L promoter alone, which is currently in Ad5-TV-CU. Replacement of the L promoter in Ad5-TV-CU with LW is likely to result in the generation of a new Ad5-TV-CU that is able to express higher levels of CD/UPRT, leading to more prostate cancer cell death, however due to time constraints I was unable to complete this part of the project.

Previous studies that have utilised prostate tissue specific promoters to drive transgene expression have ultimately failed due to poor efficacy in preclinical studies. Many of these studies have focussed around utilising specific regulatory regions of the PSA promoter, for example the PSA promoter region alone, Lu et al constructed a virus whereby E1 gene expression was under the control of the *PSA* promoter (Lu et al. 2013). They found that the virus inhibited PCa cell growth *in vitro*, detected transgene expression in xenograft tissues after infection and demonstrated inhibition of PCa tumour growth *in vivo*. Despite this the PSA promoter alone is not strong enough to induce cell death in a clinical setting, therefore studies with chimeric promoters (a fusion of the PSA promoter and enhancer), have also been investigated. Latham et al placed nitroreductase expression under the control of an optimal PSA promoter/enhancer and demonstrated sensitisation to the CB1954 prodrug following infection of LNCaP cells *in vitro* (Latham et al. 2000). More recently, chimeric promoters that are formed from regulatory regions of the PSA and PSMA genes have been used to restrict viral replication to prostate cells, but have limitations, in that they are only active in PSA/PSMA positive, androgen independent cells (Lee et al. 2004, Cheng et al. 2004). All have ultimately failed. From the data produced in this project, including the superior promoter activity of *TMPRSS2* in comparison to the previously validated *PSA* and *PSA* promoter/enhancer and enhanced cell killing with the combination of Ad5-TV-CU and 5-FC, I propose that Ad5-TV-CU could have greater cell killing potential than any of the previously published

adenoviruses, whereby suicide transgene expression is under the control of a prostate specific promoter. This could be further adapted by incorporation of the optimal LW promoter into the expression cassette to drive even higher levels of transgene expression for prostate cancer specific cell death. Ad5-TV-CU may also be effective in the apocrine ER- AR+ subtype of BCa. Apocrine BCas do not benefit from endocrine or Her2 targeted therapies, necessitating novel treatments that take advantage of AR expression.

7.1.1 Ad5-TV-CU enhances cell killing *in vitro* in combination with 5-FC in AR-positive PCa cell lines

Significant increased cell killing was observed in 22RV1, LNCaP-104-S, LNCaP-CDXR3 and LNCaP-104R1 cells when treated with a combination of Ad5-TV-CU and 5-FC prodrug. To demonstrate the target cell specificity of Ad5-TV-CU, 5-FC prodrug sensitization assays were also performed in a panel of AR-negative cell lines, including normal primary prostate epithelial cells (PrEC), Immortalized normal prostate cells (PNT1A) and the metastatic prostate cell line PC3. No increased cell killing was observed in the AR-negative cells. In fact the highest viral dose (10^5ppc) killed only ~20% of cells. This was believed to be due to the lack of CD/UPRT protein expression, which is intrinsically cytotoxic to the AR-positive cell lines in which it was translated. Additionally PC3 cells are extremely insensitive to most cytotoxic agents, including adenovirus (Oberg et al. 2010).

Despite proven efficacy of Ad5-TV-CU in 4/6 AR-positive PCa cell lines tested (22RV1, LNCaP-CDXR3, LNCaP-104R1 and LNCaP-104-S), these results could not be replicated in AR-positive LNCaP in our lab (ATCC) or VCaP cells. CD/UPRT protein was expressed in VCaP cells in a dose dependent manner following Ad5-TV-CU infection. However, no increased cytotoxicity was observed in prodrug sensitization assays. Treatment of VCaP with either 5-FC or 5-FU alone demonstrated poor sensitivity to the drugs, with no cell death induced at the highest concentration of 5-FC and only 75% cell death at the highest concentration of 5-FU (1 mg/ml), suggesting VCaP cells are resistant to 5-FU and therefore Ad5-TV-CU may have been ineffective. Resistance of VCaP cells to 5-FU was proposed to be due to the slow doubling time of this cell line (5-6 days), requiring a 6 day incubation with 5-FU for therapeutic efficacy. On one occasion, a prolonged 6 day treatment of VCaP cells with 5-FU induced a higher percentage of cell killing, suggesting that prolonged treatment of this cell line with Ad5-TV-CU

in combination with 5-FC could potentially increase 5-FC dose-dependent cytotoxicity. Furthermore, VCaP cells have a homozygous p53 mutation (A248W), that may modulate resistance to 5-FU. In fact, previous studies have described resistance to 5-FU in HCT116p53$^{-/-}$ colorectal cancer cells (Bunz et al. 1999). Therefore, a combination of the slow doubling time of the cells, coupled with the mutational background, may affect their propensity to undergo cell death.

Interestingly, LNCaP cells in our lab (ATCC) did not express CD/UPRT after Ad5-TV-CU infection, despite proven AR protein expression (section 4.2.1.1). Very low levels of dose dependent expression could be observed in one experimental repeat out of 3, and no increased cytotoxicity was observed in prodrug sensitization assays. The levels of AR expression in this cell line are lower than LNCaP-CDXR3 and LNCaP-104R1, but similar to the parental LNCaP-104-S cells. CD/UPRT expression was clearly observed in the LNCaP Chicago sublines with doses of Ad5-TV-CU as low as 2000 ppc. However, neither similar nor increasing doses of Ad5-TV-CU could induce clear CD/UPRT expression in LNCaP in our lab. This lead to STR profiling of all four LNCaP sublines, that revealed differences of the 8 core STR markers between the cell lines. There were mutational differences in 3/8 markers between LNCaP-104-S and LNCaP (ATCC). This might be sufficient to induce a differential responses to Ad5-TV-CU infection, as LNCaP (ATCC) could be missing additional transcription factors that are essential for the transcriptional activation of TMPRSS2 AREs, for example CHD8 (Menon, Yates, and Bochar 2010) or GATA2 (Perez-Stable, Pozas, and Roos 2000) (Figure 84).

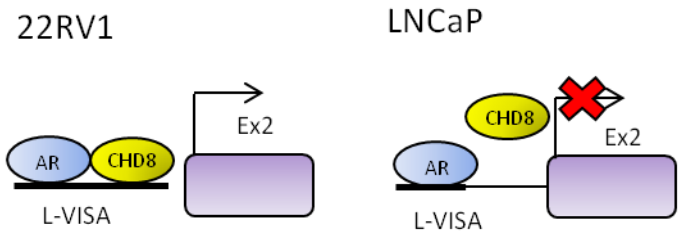


Figure 84 AR co-regulators necessary for activation of AR dependent genes

In order for AR-dependent transcription to take place a full repertoire of coactivators are required for activation, for example CHD8. Not only can these coactivators be specific for AR dependent activation, but they can also be specific to a cell line.

Previous studies have highlighted the upregulation of androgen responsive genes, including PSA and TMPRSS2, upon treatment with the synthetic androgen mibolerone in LNCaP cells (Lin et al. 1999). Efforts to demonstrate the androgen

responsiveness of the TMPRSS2 promoter in Ad5-TV-CU have been unsuccessful. No increase or decrease in CD/UPRT transgene expression could be detected upon addition of either mibolerone or Bicalutamide, leading to questions surrounding the androgen responsiveness of Ad5-TV-CU. However, I performed these studies solely in 22RV1 cells, with a constitutively active AR. This constitutive activation renders AR insensitive to activation by additional androgens and unresponsive to antagonists, therefore the AREs in the *TMPRSS2* promoter sequence present in Ad5-TV-CU are unresponsive to the androgen bound receptor, or androgens do not bind to the receptor in 22RV1 cells. Further studies in androgen-dependent cell lines, for example AR transfected CHO cells, in combination with utilising more sensitive methods for detecting changes in expression levels, for example a luciferase reporter assay, would confirm whether this was the case. In addition these experiments would demonstrate the androgen responsiveness of the *TMPRSS2* promoter elements present in Ad5-TV-CU.

7.1.2 Combination of L and W AREs from TMPRSS2 drive high specific expression in AR-positive cell lines

Both the L and W promoters are incapable of driving high levels of prostate specific transgene expression alone. The increased specificity of the L-promoter in Ad5-TV-CU, that contains four out of six AREs located in the PSA enhancer, lead to questions surrounding whether transgene expression from the *TMPRSS2* promoter regions could be increased through a combination of multiple androgen responsive regions. Therefore, I combined the L and W-AREs and found that this combination increased both luciferase transgene expression and specificity (section 6.2.2.2). Furthermore, previous studies have demonstrated that combining multiple androgen responsive regions increases transgene expression (Latham et al. 2000). Li et al demonstrated that expression of viral E1A and E4 genes could be tightly controlled by the PSES chimeric enhancer, containing enhancer elements from PSA and (Li et al. 2005). The efficacy of this virus was tested *in vivo*, and showed that the growth of CWR22rv xenografts was greatly inhibited compared to tumours treated with a control virus. However, this is only effective in PSA/PSMA-positive androgen-independent cell lines, as PSMA is inhibited by androgens, and viral replication slowed after several days. Similarly, Ahn et al used the PSA/PSMA chimeric promoter to restrict a replicating adenovirus armed with the herpex simplex virus thymidine kinase, called AdIU1, to treat CWR22rv tumour xenografts and found a stronger therapeutic effect with the

combination Of AdIU1 and GCV, compared to AdIU1 alone (Li et al. 2005, Ahn et al. 2009). These cases provided further evidence for the hypothesis that combining multiple androgen responsive regions, including promoters and enhancers could increase transgene expression.

Three models have been suggested that could explain the interaction between a proximal promoter and a distal enhancer, including the tracking model, the linking model and the looping model. The tracking model suggests that signals are recruited by enhancers through the DNA to promoters and the linking model suggests that the looped enhancer-promoter interaction is due to propagation of nucleoprotein structures along the intervening DNA. The looping model suggests that proteins that are directly bound to either enhancers or promoters interact with each other (Bulger and Groudine 1999). In fact, Wang et al demonstrated, using ChIP-3C methodology, that recruitment of AR and its essential coactivators to the W region and the upstream W enhancer resulted in the formation of chromatin looping that allowed RNA polymerase II to track from the enhancer to the promoter (Wang, Carroll, and Brown 2005). Consequently, I initially combined both the L and W AREs with the previously published *TMPRSS2* enhancer (WE), located -13.5 kb upstream from the *TMPRSS2* promoter (Wang et al. 2007). However, combining these sequences did not increase transgene expression in my hands; in fact it had very little effect on expression levels from the vector, possibly due to the close proximity of the sequences. A further combination of the L-ARE (upstream of Exon1) and W-ARE (upstream of Exon2), creating the LW chimeric promoter, not only drove higher levels of expression than the W-ARE alone, but restored prostate specificity to the vector, resulting in higher levels of prostate specific transgene expression and a new optimal promoter. I therefore hypothesise that the L and the W regions interact, through chromatin looping, in a similar fashion to that identified by Wang et al, in order to drive higher levels of prostate specific expression (Figure 85).

It will be important to establish the AR dependency of both the L and W regions for future studies, simple experiments to demonstrate this could include comparison of luciferase expression in LW-PGL3 transfected PC3 and DU145 (AR negative) cell lines co-transfected with an AR expressing plasmid vs without, as well as transfection of 22RV1 cells with LW-PGL3 ± AR siRNA to demonstrate the effect of inhibiting AR expression on the activation of LW-PGL3 using traditional luciferase promoter assays.

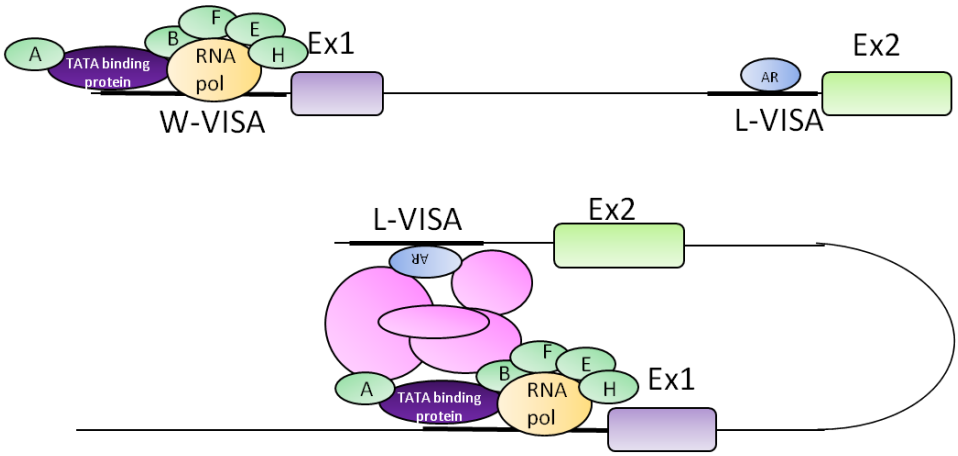


Figure 85 Proposed model for the interaction of L and W regions through chromatin looping

Transcription factors bind to both the L and W regions of the *TMPRSS2* gene, these regions then interact with each other through chromatin looping to initiate transcription.

7.1.3 Ad5-TV-CU failed to prevent xenograft growth due to poor animal models *in vivo.*

A number of different animal models and cell types were utilised to investigate the efficacy of Ad5-TV-CU *in vivo* (

Table 31). Whilst all three studies testing Ad5-TV-CU *in vivo* showed a trend towards decreased tumour growth after treatment, there was no clear differentiation between the control group treated in combination with PBS, and the target group treated in combination with 5-FC. The main reason for this in the LNCaP-CDXR3 cells and LNCaP-104-S studies was poor tumour growth. Some causes for the poor tumour growth include; testosterone levels in the animals, immune infiltration and physical manipulation of the tumours. Attempts to develop a better model by eliminating the addition of testosterone and using animals with more complete immunodeficiency (NOD/SCID) also failed to generate a suitable *in vivo* model. Tumours did not develop in NOD/SCID mice bearing 1.25 mg subcutaneous testosterone pellets and grew both slowly and erratically in the animals without testosterone pellets, suggesting a more complex affect of testosterone on the development of LNCaP-104-S tumours than previously thought. The final model, CD-1 mice bearing 22RV1 xenografts developed much faster than both the LNCaP-CDXR3 and LNCaP-104-S (within two weeks compared to 5-6 weeks for LNCaP-CDXR3 and 4 weeks for LNCaP-104-S). All

untreated control animals with 22RV1 xenografts had to be culled 2 weeks later as a result of tumour burden due to the accelerated growth of these tumours compared to the LNCaP sublines. However, tumours treated with Ad5-TV-CU in combination with either PBS or 5-FC in the remaining animals progressed much slower. Unfortunately there was no significant difference in tumour growth between the two groups treated in combination with either 5-FC or PBS, suggesting that Ad5-TV-CU alone, or potentially physical manipulation of the tumour is responsible for inhibited tumour growth. Therefore, a more appropriate animal and tumour model would need to be established to truly determine the *in vivo* efficacy of Ad5-TV-CU in combination with 5-FC.

7.1.4 Realistic summary of the findings

There were a number of points throughout this study where careful consideration should have been taken over decisions in order to avoid larger problems accumulating further down the line, most noticeably the discovery of the PSA enhancer in the VISA system. When we received the VISA vector we were given a plain plasmid map with very little annotation. We were aware that the system had been used to control luciferase expression through the CCKAR promoter and were given the positions of the restriction enzymes needed to cut out both CCKAR and luciferase, but were unaware of what else remained in the plasmid. Once received, the entire plasmid should have been immediately sequenced and run through UCSC genome browser in order to confirm exactly what the plasmid contained. Alternatively we should have questioned professor Hung when we realised that there was ~700 bp of sequence unaccounted for in order to learn more about the history of the plasmid. After further investigation later on in my study I identified that the plasmid was obtained from Zhang and they had previously been working with PSA enhancer constructs in the VISA vector. The discovery of the PSA enhancer led to many questions over the contribution it gave to transgene expression levels and questions surrounding the entire foundation of the project, as it was already present in Ad5-TV-CU. Although the potential effects of the PSA enhancer and *TMPRSS2* promoters were investigated at a later stage of my study, if the PSA enhancer had been discovered earlier, the investigations into the *TMPRSS2* promoter regions would have started at an earlier stage in my project. This would have resulted in the best combination of the promoter elements, (LW) being used for the *in vitro* and *in vivo* investigation of therapeutic efficacy. Had this been the case, the replicating virus containing the LW promoter

could have been made along with a number of controls to properly assess the transgene expression.

Despite the identification of the PSA enhancer in VISA it also would have been beneficial to produce a control virus that did not contain the VISA expression cassette, whereby CD/UPRT expression was simply controlled by the *TMPRSS2* promoter in order to see the true amplification affect of VISA. Xie et al. demonstrated that the VISA system dramatically amplified the CCKAR promoter activity in AsPC-1, PANC-1 and Panc02 cells by 826-, 256- and 353-fold respectively (Xie et al. 2007). However, it would have been difficult to directly compare expression levels of the two viruses due to interference from the existing PSA enhancer, therefore inactivation of this region from the VISA vector via site directed mutagenesis or deletion would be beneficial for future studies. Alternatively, an identical control virus whereby CD/UPRT expression is under the control of either a CMV or PSA promoter would also be beneficial in determining the true specificity and strength of the *TMPRSS2* promoter.

Furthermore, Ad5-TV-CU, containing the L-ARE alone, has cell killing abilities *in vitro* in combination with 5-FC in 22RV1 and LNCaP-104S, as well as in the castration resistant cell lines, LNCaP-CDXR3 and LNCaP-104R1. However, this efficacy was not comparable to that which has been seen using similar GDEPT systems in replicating viruses at much lower multiplicity of infection (MOI). Dias et al induced 40 and 90% cell killing in UT-SCC and UT-SCC-29 cells respectively in combination with 500 µg/ml 5-FC at an MOI of 1 (Dias et al. 2010). In contrast 4000 ppc of Ad5-TV-CU in combination with 500 µg/ml was required to kill 95% of 22RV1 cells in my hands. It could be argued that this may be due to the replication-deficient nature of Ad5-TV-CU. However Erbs et al. induced 80, 95 and 80% cell killing in SW480, SK-BR-3 and Panc-1 human tumour cells respectively, with a non-replicating adenovirus expressing CD/UPRT at MOI of 5 in combination with 500 µg/ml 5-FC (Erbs et al. 2000), further demonstrating that Ad5-TV-CU is not as effective at cell killing in combination with 5-FC as some previously validated adenoviral vectors. It is however worth noting that these viruses used the constitutively active CMV promoter and are therefore not directly comparable to Ad5-TV-CU. It is not expected that a tissue specific promoter would outcompete the CMV or SV40 promoters and nor do they drive tissue specific expression, rendering them inadequate for clinical use in this instance. Therefore, the LW promoter shows promise as it demonstrated both increased expression and high

selectivity, warranting further investigation of this sequence and continuation of the project in the future.

Finally, *in vivo* studies failed to demonstrate tumour growth inhibition in combination with 5-FC, due to a number of technical difficulties including poor animal models, inefficient controls (Ad5-GFP induced too much toxicity alone in the initial pilot studies), the replication-deficient nature of the virus preventing intratumoural spread, and other factors resulting in poor or no efficacy. In its current format non-replicating Ad5-TV-CU is not efficacious enough to be translated into the clinic. However, the ultimate goal of my work presented in this thesis, in combination with future findings guided by this study, is to more fully understand the AR transcriptional control of the *TMPRSS2* promoter. Once this is understood I hope these regions could be utilised to drive prostate specific expression in a replicating virus, which will be translated into the clinic to treat patients with both primary PCa, and castration resistant forms of the disease.

In conclusion, I have identified an optimal *TMPRSS2* chimeric promoter (LW), which specifically drives luciferase expression in AR-positive 22RV1 cells, whilst remaining inactive or driving minimal expression in AR-negative cell lines. The LW promoter induced 8.9-fold higher expression levels than the L-ARE alone (already present in Ad5-TV-CU). Incorporation of this optimal promoter into future targeted therapies may aid further developments in the treatment of PCa, with potential applications in forms of aggressive AR-positive BCa.

7.2 Future directions

Once cloned into the VISA vector, the LW promoter should be capable of driving similar expression levels to the constitutively active CMV promoter. Cloning of this new optimal expression cassette into a replication-competent adenovirus will dramatically increase efficacy of the virus and is more likely to induce cytotoxicity in xenografts *in vivo* in combination with 5-FC. This is due to the increased cell killing potential of replicating viruses and has been demonstrated in numerous clinical trials that have utilised replicating viruses, over replication deficient viruses to target PCa (Freytag et al. 2003, DeWeese et al. 2001).

Whilst modifications to the virus are essential for producing an efficacious gene therapy system, careful selection of a model in which to test the virus *in vivo* is also imperative. It is possible that future *in vivo* studies, including novel AR expressing cell lines, patient ex vivo specimens or alternative model systems for example zebrafish will allow a more clear idea of the full potential for Ad5-TV-CU as a treatment for PCa. Combinatorial therapies with other cytotoxic drugs may also enhance the activity of this virus *in vivo* and present a more effective treatment with potentially groundbreaking results for the treatment of both localised and metastatic PCa in the clinic.

Therefore, future studies must focus on utilising the optimal LW promoter in a replication-competent virus. These studies should initially involve inserting the LW-promoter into the E1A region of the adenovirus so that viral replication is under the control of the *TMPRSS2* promoter. This will demonstrate the prostate specificity of viral replication without interference from the rest of the expression cassette. Once prostate specificity has been established the CD/UPRT transgene can be inserted downstream to evaluate enhanced cell killing of the virus in combination with 5-FC prodrug. Previous studies have shown that 5-FU and 5-FUMP interfere with viral replication (McCart et al. 2000, Nakamura et al. 2001, Dias et al, 2010). However, Dias et al showed that although the addition of 5-FC reduced adenoviral replication 9.2-fold, the combined oncolytic efficacy was greater with the addition of 5-FC than without, with a 27% increase in cell killing in combination. Therefore careful consideration needs to be taken in deciding whether to use this CD/UPRT GDEPT approach in the new replicating adenoviral construct, or whether the

oncolytic virus driven specifically by the *TMPRSS2* promoter elements will be enough to significantly increase cell killing compared to the non-replicating virus.

This can be achieved as follows:

- Identifying the minimal regions of the LW promoter that retains potency and selectivity.
- Inserting the optimal promoter into the E1A region of the adenovirus to control viral replication and evaluate specificity and efficacy of the *TMPRSS2* promoter alone.
- Inserting CD or CD/UPRT downstream of the *TMPRSS2* promoter in the replicating virus (Ad5-TV-CUrep) to evaluate the efficacy of the two prodrug systems in combination with 5-FC. This would include experiments to evaluate whether the timing of 5-FC addition affects the cell killing potential of the virus/ viral replication.
- Testing the new Ad5-TV-CUrep again in all cell lines, including our LNCaP (ATCC) cells to see if efficacy can be generated with the new virus.
- Testing Ad5-TV-CU *in vivo* in combination with 5-FC and other cytotoxic drugs to enhance cell killing.
- Identifying a suitable *in vivo* model in which to test the tumour inhibition capabilities of Ad5-TV-CU in combination with 5-FC and cytotoxic drugs that have demonstrated synergistic effects on cell killing *in vitro*.

Additionally, it will be important to explore in depth, the AR-dependency of the various *TMPRSS2* promoter/enhancer constructs and how they interact. The *TMPRSS:ERG* fusion gene is the most common fusion gene in PCa, occurring in 50% of PCas. It is not yet clear whether the fusion gene contributes to the progression of aggressive forms of PCa, and arguments exist for and against (Rajput et al. 2007, Eguchi et al. 2014). Therefore, investigating and determining the contribution of AR-dependent regions of the *TMPRSS2* promoters identified in this project, may reveal the true nature and role of the *TMPRSS2:ERG* fusion. Findings from this future work may guide the development of more effective therapies for late stage PCa that harbour the gene fusion.

This could be achieved by the following:

- Comparison of luciferase expression levels in LW-PGL3 transfected PC3 and DU145 (AR negative) cell lines co-transfected with an AR expressing plasmid vs without.
- Transfecting 22RV1 cells with LW-PGL3 ± AR siRNA.
- Demonstrating the interaction between the L and W regions via chromatin looping through ChIP-3C assays.
- Co-transfecting AR negative cells with a plasmid expressing AR as well as the various TMRPSS2 promoter/enhancer constructs and measuring luciferase expression as a result.
- Infecting cells that harbour wildtype AR with the new Ad5-TV-CUrep virus, in combination with a repertoire of AR agonists and antagonists to establish the effect on CD/UPRT or luciferase expression.
- Demonstrating AR binding to the putative AR binding sites in both the L and W regions through ChIP assays.

CHAPTER EIGHT

APPENDIX

8.1 Sequencing

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PW-PGL3

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W-prom enhancer-PGL3

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EL-PGL3 sequencing

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LW-PGL3 sequencing

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Sequences of the L-VISA and W-VISA promoter regions present in the plasmids. underlined regions show AREs:

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Lin Paper, identification of putative ARE and promoter of TMPRSS2

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8.2 *In vivo*

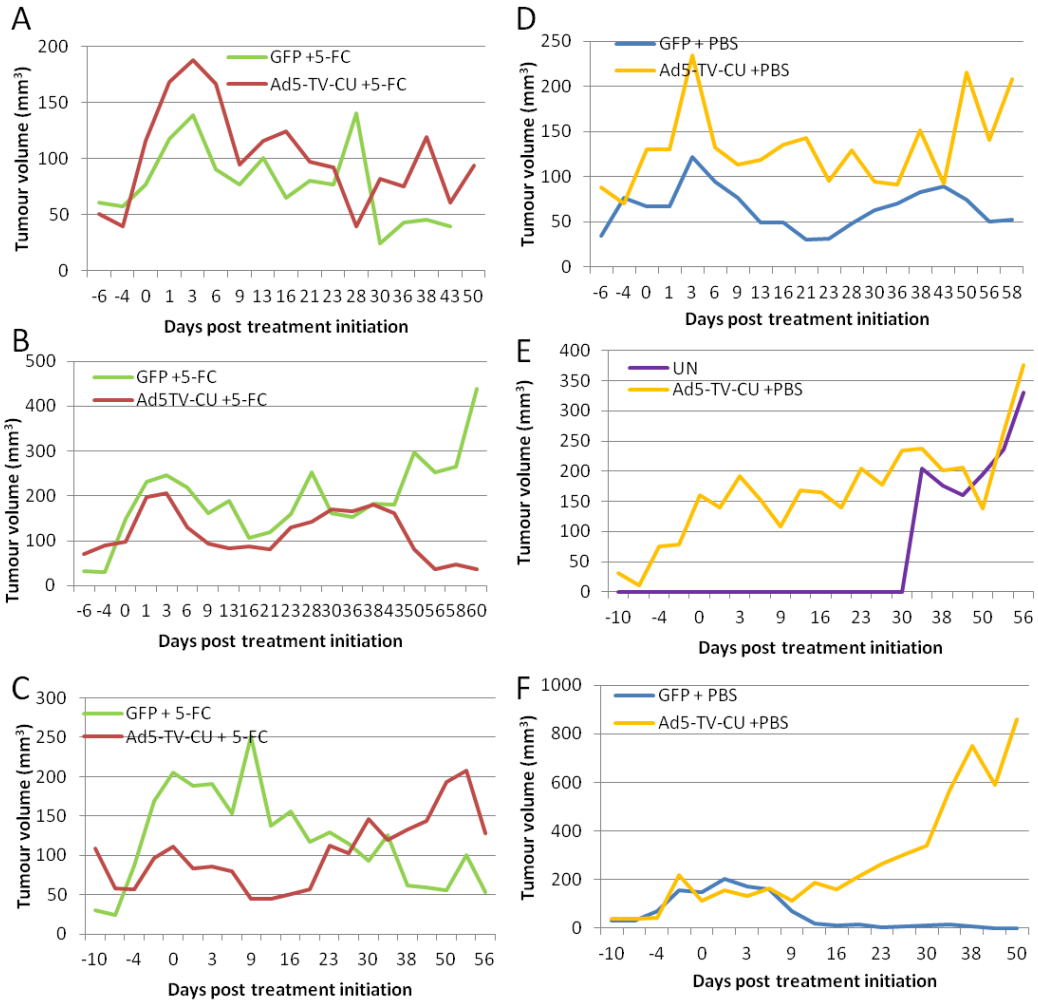
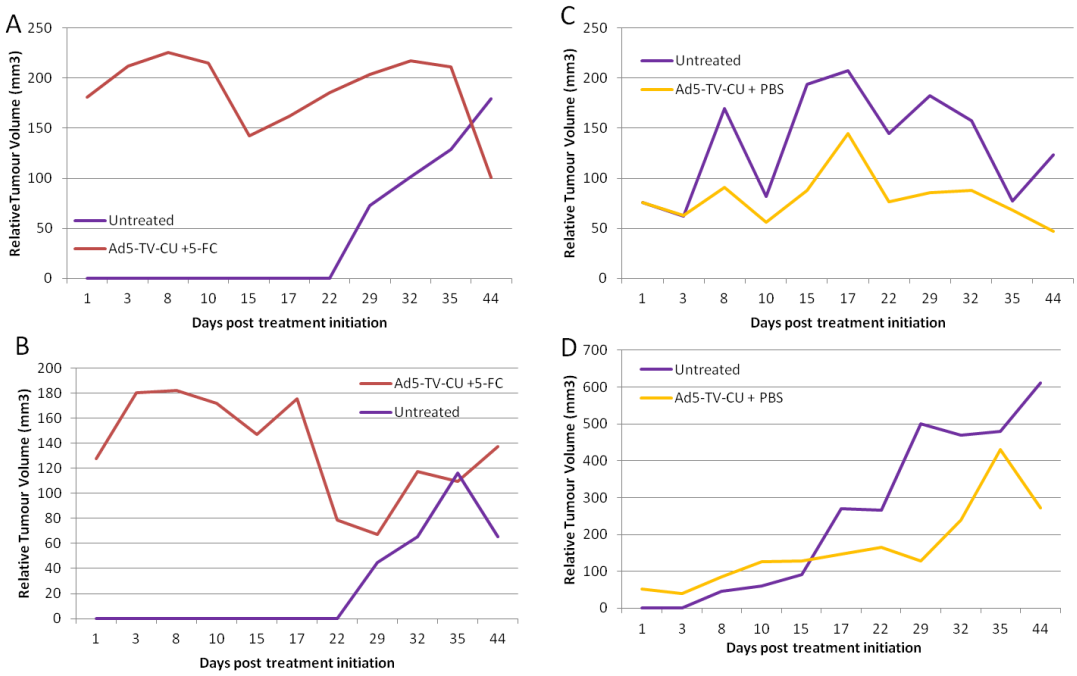


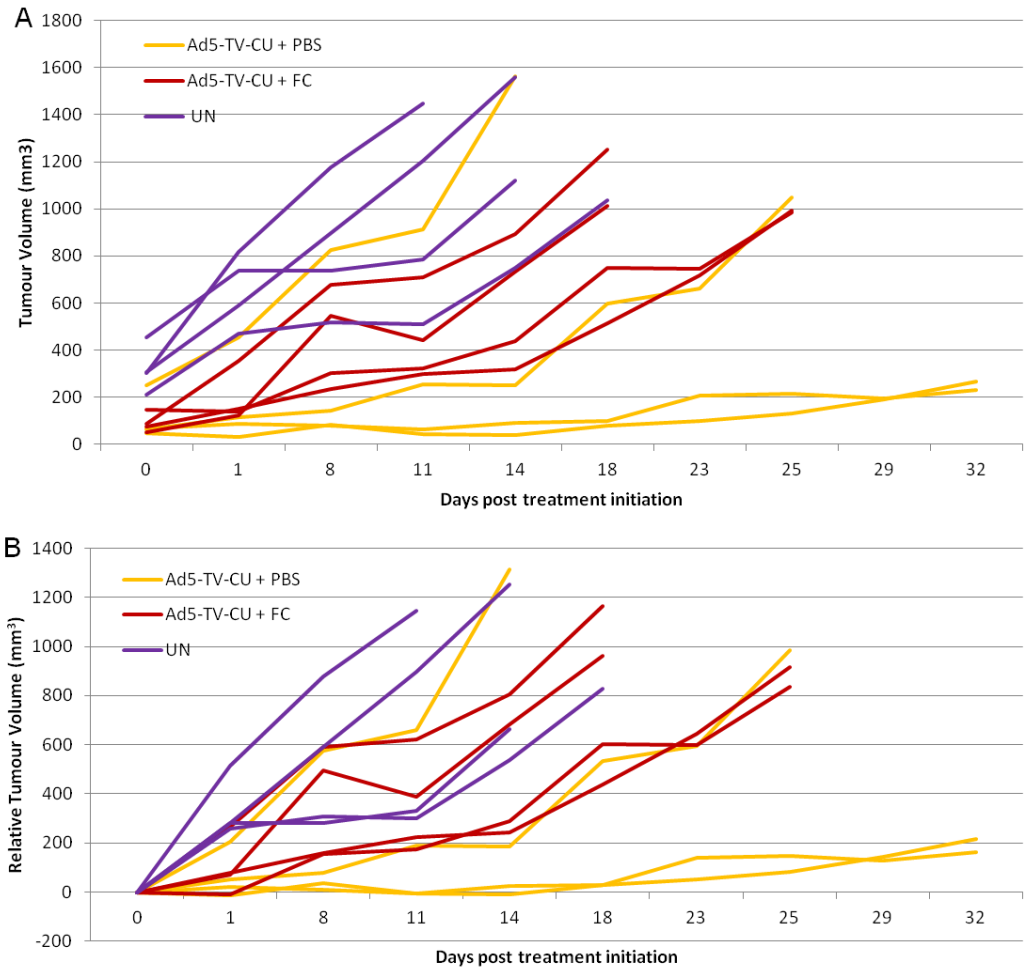
Figure_ Apx1 Graphs showing LNCaP-CDXR3 tumours in individual animals (on the left and right flanks)

Male BALB/c castrated mice were injected subcutaneously with 1 x10^6 hormone independent LNCaP-CDXR3 cells in 50% matrigel. After allowing tumours to grow for 5 weeks to 100 mm^3 mice were separated into groups treated with Ad5-TV-CU in combination with 5-FC or Ad5-TV-CU in combination with PBS and the day designated day 1. Graphs show LNCaP-CDXR3 tumours on the left and right flanks of individual mice. **A, B** and **C** show mice treated with 5-FC in combination with Ad5-TV-CU (red lines) or Ad5-GFP (green lines). **D, E** and **F** show all animals treated with PBS in combination with Ad5-TV-CU (yellow lines) or Ad5-GFP (blue lines). One mouse developed a second tumour 30 days post treatment initiation, which was left untreated (purple line). Tumours were measured relative to the starting tumour volume on day 1 of treatment,



Figure_Apx2 Graphs showing LNCaP-104-S tumours in individual animals (on the left and right flanks)

Male BALB/c mice were injected subcutaneously with 1 x10^6 hormone dependent LNCaP-104-S cells in 50% matrigel in combination with a 1.25 mg testosterone pellet. After allowing tumours to grow for 5 weeks to 100 mm^3 mice were separated into groups treated with Ad5-TV-CU in combination with 5-FC or Ad5-TV-CU in combination with PBS and the day designated day 1. Intratumoural injections of Ad5-TV-CU were administered on days 1, 3 and 6 and intraperitoneal injections of either 5-FC or PBS given on days 2, 5, 8 and 15.Graphs show LNCaP-104-S tumours on the left and right flanks of individual mice. **A** and **B** show mice treated with Ad5-TV-CU in combination with 5-FC (red lines) or untreated tumours (purple lines) that developed 22 days post treatment initiation. **C** and **D** show all animals treated with Ad5-TV-CU in combination with PBS (yellow lines) or untreated tumours (purple lines), that developed a number of days after treatment initiation. Tumours were measured relative to the starting tumour volume on day 1 of treatment,



Figure_Apx3. Tumour growth was inhibited in 22RV1 tumour xenografts treated with Ad5-TV-CU in combination with 5-FC and PBS

Male CD-1 athymic mice were injected subcutaneously with 1 x10^6 hormone independent 22RV1 cells in 50% matrigel. After allowing the tumours to grow for 2 weeks to ~100 mm^3 mice were separated into the control groups, Untreated (purple lines), Ad5-TV-CU plus PBS (yellow lines), or target group, Ad5-TV-CU plus 5-FC (red lines) The first tumour measurement was designated day 0. Intratumoural injections of Ad5-TV-CU were administered on days 1, 3 and 6 and intraperitoneal injections of either 5-FC or PBS given on days 2, 5, 8 and 15. **A.** Data shows the tumour volume in mm^3 up to day 32, when the study was terminated. **B.** Data shows relative tumour volume post treatment initiation. Tumour volume was normalised relative to the individual starting tumour volume determined on day 0 to illustrate the effect of treatment on tumour volume. n= number of tumours per group. n=4 (Ad5-TV-CU+5-FC, Ad5-TV-CU+PBS, UN).

CHAPTER NINE

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