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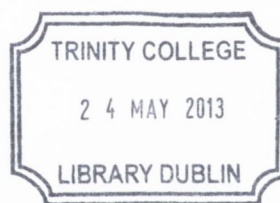
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In vitro modelling of androgen-mediated gene-
directed enzyme prodrug therapy for the treatment
of prostate cancer

A thesis submitted for the degree of
Doctor of Philosophy

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B.A. (Genetics)

University of Dublin, Trinity College
December 2012

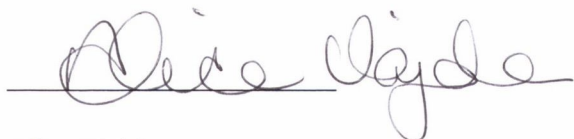


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December 2012

Summary

Gene-directed enzyme prodrug therapy has the potential to improve prostate cancer therapy outcome and could be used on its own or as an adjuvant to the current treatments. This research involved the characterisation of a vector previously created in the Prostate Molecular Oncology Research Group, pPPE-CD, in which the promoter and enhancer of prostate specific antigen (PSA) gene had been cloned upstream of the gene for cytosine deaminase (CD). CD is an enzyme that converts a drug with relatively low toxicity, 5-fluorocytosine (5-FC) to its active form, 5-fluorouracil. This strategy may allow for specific targeting of prostate tumour cells as CD expression is restricted by androgen levels, which bind to the androgen receptor (AR), activating it and upregulating PSA expression.

Initial experiments involving aerobic (21% oxygen) and hypoxic (0.5% oxygen) cell samples and qRT-PCR indicated that careful selection of the endogenous control gene is necessary as hypoxia affects the expression of a multitude of genes. This led to a study involving 16 endogenous control genes and the assessment of their variability in aerobic and hypoxic samples from four prostate cancer cell lines and two normal prostate cell lines. The disparity between cell lines in endogenous control gene expression demonstrated that verification of the endogenous control is a necessary step before beginning qRT-PCR with any new cell line or treatment condition. The importance of correct endogenous control selection was demonstrated by calculating the expression levels of *HIF-1 α* when some of the best and worst performing genes were used as controls.

Further characterisation of the activity of the pPPE-CD vector demonstrated that cross-talk between HIF-1 and AR leads to higher levels of CD expression in cells exposed to 0.5% oxygen compared to 21% and consequently higher levels of cell kill when 5-FC is administered. This is of interest as targeting of hypoxic cells within a tumour may be advantageous as these cells have been shown to be resistant to common treatments, including radiotherapy and chemotherapy. Further study of the activity of pPPE-CD found CD expression levels were increased when androgen stimulation was combined with a dose of radiation. This was shown to correlate with increased expression of the

androgen receptor. This research suggests the pPPE-CD/5-FC system has potential as an adjuvant to radiation therapy which could result in improved outcome.

22Rv1 cells are a widely used model of primary prostate cancer but their level of PSA expression does not correlate with the high levels typically seen *in vivo*. In order to better mimic clinical PSA levels, a new stably transfected 22Rv1 cell line was generated with overexpression of AR to create a model that would allow further testing of the activity of pPPE-CD in an environment closer to *in vivo* conditions. The stable transfection was successful but surprisingly this led to decreased PSA expression compared to the parent cell line. This significant downregulation in expression was not observed when levels of *FKBP51*, another AR regulated gene, were tested. Although the wild type AR was overexpressed in 22Rv1, an increase in the truncated as well as the full length isoform was observed. It has been demonstrated that the two AR isoforms differ in terms of the genes that they regulate, which could be a possible reason for the observed PSA results. However, calpain activators and inhibitors which decreased and increased the relative levels of the truncated isoform of AR did not influence PSA expression.

A second attempt was made to create an improved *in vitro* model of prostate cancer using a novel suspension medium, 'Happy Cell', developed in Trinity College. Optimisation of the technique led to the creation of 22Rv1 spheroids. To assess the utility of this model for analysis of pPPE-CD, a GFP-expressing vector was used to establish that transfected cells could form spheroids and maintain expression for at least 7 days. Of note, PSA expression was measured and found to be significantly higher for the 22Rv1 spheroids compared to those grown as an adherent monolayer. pPPE-CD transfected spheroids further demonstrated the potential of this GDEPT system in response to 0.5% oxygen and radiation treatment. The results suggest that spheroids may be an improved *in vitro* model with great potential for future investigation of prostate cancer and its treatment, and that the pPPE-CD/5-FC GDEPT system has clinical potential in the treatment of prostate cancer.

Acknowledgements

I would like to thank Laure Marignol, my supervisor, for advising and helping me throughout this process, even while on maternity leave! I am very grateful for the invaluable guidance she gave. Ruth Foley and Antoinette Perry were also a really great help and very supportive. I would like to express my appreciation to the P.I of the Prostate Molecular Oncology group, Prof. Donal Hollywood, for his encouragement and help with the direction of the project.

Lynn Martin and Therese Murphy gave me great encouragement, advice and pep-talks. I would also like to thank the other members of the Prostate group as well as the Durkan Lab past and present; Armelle, Tony, Elaina, Linda, Prerna John, Colm, Eva, Fardod, Diarmuid, James and Derek. I am grateful to other researchers from cell culture who were always willing to share the wealth of their knowledge throughout my PhD, especially Martin, Anne-Marie and Niamh.

My family and friends have been a great support throughout my studies. I would especially like to thank Russell McLaughlin as without him, I would probably not have reached the end with my sanity still intact!

Publications

Published

“Clinical potential of gene-directed enzyme prodrug therapy to improve radiation therapy in prostate cancer patients.”

Cancer Treatment Reviews. 2011 Dec;37(8):643-54. Epub 2011 Apr 9.

Gene expression analysis in prostate cancer: the importance of the endogenous control. The

Prostate 2012 Aug 27. doi: 10.1002/pros.22578. [Epub ahead of print]

Presentations

Oral

Irish Association for Cancer Research Conference (Feb 29th – March 2nd 2012) - Belfast

Poster

Institute of Molecular Medicine Research Day (November 2010) - Dublin

Institute of Molecular Medicine postgraduate research day (September 2010) - Dublin

Irish Association for Cancer Research Conference (3rd – 5th March 2010) – Galway

European Society of Gene and Cell Therapy (November 2009) – Hannover, Germany

Institute of Molecular Medicine Research Day (November 2009) - Dublin

International Society of Cell and Gene Therapy (September 2009) – Cork

All Ireland Cancer Conference (13th – 15th May 2009) – Dublin

Irish Association for Cancer Research Conference (5th – 6th March 2009) – Meath

All Ireland Cancer Conference (Nov 30th -Dec 3rd 2008) – Dublin

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Travel Award for the Irish Association for Cancer Research Conference in 2009 and 2010

Winner of the 2009 Poster Prize at International Society of Cell and Gene Therapy conference

Contents

Declaration..... i

Summary iii

Acknowledgements..... v

Publications..... vii

Presentations vii

Awards viii

Contents ix

Tables..... xvii

Figures xviii

Abbreviations..... xxv

Chapter 1 Introduction..... 3

 1.1 The prostate gland 3

 1.1.1 Structure..... 3

 1.1.2 Function 3

 1.2 Prostate cancer 4

 1.2.1 Epidemiology 4

 1.2.2 Symptoms 5

 1.2.3 Progression..... 6

 1.2.4 Diagnosis 6

 1.3 The Androgen Receptor 7

 1.4 Hypoxia 10

 1.5 Current treatments for prostate cancer 13

 1.5.1 Management of localised prostate cancer 13

 1.5.2 Management of local failure following therapy 16

ix

1.5.3	Management of metastatic prostate cancer	17
1.5.4	Neoadjuvant/adjuvant therapy	18
1.6	Gene directed enzyme prodrug therapy	20
1.6.1	Cytosine deaminase/5-fluorocytosine prodrug system	21
1.6.2	Herpes simplex virus thymidine kinase/ Ganciclovir prodrug system	22
1.6.3	Other enzyme/prodrug systems.....	23
1.7	GDEPT delivery vectors	24
1.8	Optimal adenoviral delivery	24
1.9	Combining GDEPT with radiation therapy.....	25
1.9.1	Radiosensitization	25
1.9.2	Improved viral uptake	26
1.9.3	GDEPT for the management of local failure following radiation therapy 27	
1.9.4	GDEPT to improve radiosensitivity in newly diagnosed localised prostate cancer 30	
1.9.5	GDEPT for the treatment of metastatic prostate cancer	33
1.10	Transcriptional targeting of GDEPT	37
1.10.1	Prostate-specific	37
1.10.2	Hypoxia-responsive	39
1.10.3	Radiation-inducible.....	39
1.11	Hypothesis & project aims	41
Chapter 2	Materials and Methods.....	45
2.1	Cell culture	45
2.1.1	Cell lines	45
2.1.2	Growth conditions.....	45

2.1.3	Coating flasks with poly-D-lysine	45
2.1.4	Cell subculture	45
2.1.5	Counting cells	46
2.1.6	Making cell stocks	46
2.1.7	Reconstitution of frozen cells	46
2.1.8	Mycoplasma testing	47
2.2	Hypoxia	47
2.3	Irradiation	47
2.4	Clonogenic assay	47
2.5	Vector preparation	47
2.5.1	Agar plate preparation	47
2.5.2	Transformation	48
2.5.3	Growth of bacterial cultures	48
2.5.4	DNA extraction - Miniprep	48
2.5.5	Glycerol stocks	48
2.5.6	Growth of bacterial cultures from glycerol stocks	49
2.5.7	DNA extraction - Midiprep	49
2.6	Agarose gel electrophoresis	49
2.7	CellTiter 96 [®] non-radioactive cell proliferation assay (MTT assay)	50
2.7.1	Cell number standard curve	50
2.8	Cell viability following pPPE-CD/5-FC treatment	50
2.9	PSA-EIA	51
2.10	Transfection	51
2.11	Protein collection	52
2.12	Bradford assay	52

2.13	Western blotting	53
2.13.1	Polyacrylamide gel electrophoresis	53
2.13.2	Transfer	54
2.13.3	Blotting	54
2.13.4	Loading control blotting	55
2.14	TaqMan gene expression assay	56
2.14.1	RNA extraction - RNeasy mini kit.....	56
2.14.2	RNA extraction – TRI Reagent.....	56
2.14.3	Tissue samples	57
2.14.4	DNase treatment.....	57
2.14.5	cDNA synthesis	58
2.14.6	qRT-PCR.....	58
2.14.7	Data analysis	59
2.14.8	Human endogenous control plate.....	60
2.15	siRNA	60
2.16	Creation and characterisation of androgen receptor overexpressing 22Rv1	61
2.16.1	The vector	61
2.16.2	Transformation/ DNA extraction	61
2.16.3	Agarose gel electrophoresis	61
2.16.4	Restriction digest	61
2.16.5	Glycerol stocks.....	61
2.16.6	DNA extraction- Midiprep.....	62
2.16.7	Optimisation of Geneticin concentration	62
2.16.8	Transfection	62
2.16.9	Cell cloning by serial dilution.....	62

2.16.10	Calpastatin and Ionomycin treatment (new section).....	63
2.17	Bicalutamide treatment.....	64
2.17.1	MTT assay	64
2.17.2	Clonogenic assay	64
2.18	Spheroids	64
2.18.1	Cell culture.....	64
2.18.2	Confocal microscopy	65
2.18.3	Automated liquid handling	65
2.18.4	PSA EIA	66
2.18.5	GFP transfection	66
2.18.6	pPPE-CD/5-FC toxicity	66
2.18.7	High content screening	67
Chapter 3	Gene expression analysis in prostate cancer: the importance of the endogenous control	71
3.1	Introduction	71
3.1.1	Aims and Objectives.....	73
3.2	Results	74
3.2.1	Optimal endogenous control genes for aerobic (21% oxygen) and hypoxic (0.5%) samples.....	74
3.2.2	Performance of selected endogenous control genes following longer exposure to 0.5% oxygen.....	79
3.2.3	The choice of endogenous control can result in variation in gene expression results.....	80
3.2.4	In silico analysis of hypoxia response elements	81
3.2.5	Performance of selected endogenous control genes in tumour and normal tissue	81

3.3	Discussion	83
Chapter 4	Characterisation of pPPE-CD activity under hypoxic conditions.....	89
4.1	Introduction	89
4.1.1	pPPE-CD2.15 and pCMV-CD1.10 vectors	89
4.1.2	AR/HIF-1 cross-talk.....	90
4.1.3	Aims and objectives	95
4.2	Results	96
4.2.1	Determination of optimal concentration of DHT for PSA expression.....	96
4.2.2	Investigation of AR/HIF-1 cross-talk and its effect on the activation of pPPE-CD.....	98
4.3	Discussion	117
Chapter 5	Investigation of pPPE-CD activity in combination with radiation	125
5.1	Introduction	125
5.1.1	Aims and objectives	126
5.2	Results	127
5.2.1	PSA expression in response to radiation.....	127
5.2.2	pPPE-CD expression in response to radiation	131
5.2.3	Cell viability in pPPE-CD transfected LNCaP and 22Rv1 with radiation treatment	134
5.2.4	Clonogenics assay testing pPPE-CD/5-FC with radiation treatment.....	140
5.2.5	AR expression in response to radiation	142
5.2.6	NF- κ B expression in response to radiation	145
5.3	Discussion	147
Chapter 6	Creation and characterisation of androgen receptor-overexpressing 22Rv1	155
6.1	Introduction	155

6.1.1	Aims and Objectives.....	157
6.2	Results.....	159
6.2.1	Restriction digest	159
6.2.2	Optimisation of Geneticin concentration.....	159
6.2.3	AR protein levels	160
6.2.4	Relative quantification of AR gene expression	162
6.2.5	PSA protein levels	163
6.2.6	Relative quantification of PSA gene expression.....	164
6.2.7	Relative quantification of FKBP51 gene expression.....	166
6.2.8	Growth rates.....	167
6.2.9	Efficiency of CD expression in pPPE-CD transfected 22Rv1-ARhi.....	169
6.2.10	Response to Bicalutamide treatment.....	170
6.2.11	Cytoplasmic and nuclear protein levels of AR	173
6.2.12	Calpain activation and inhibition.....	175
6.3	Discussion	183
Chapter 7	Development of a spheroid model of prostate cancer and its use to evaluate the activity of pPPE-CD	193
7.1	Introduction	193
7.1.1	Aims and objectives.....	195
7.2	Results.....	197
7.2.1	Optimisation of spheroid formation.....	197
7.2.2	Spheroids vs. aggregates.....	198
7.2.3	Morphological assessment of spheroids	199
7.2.4	Mechanism of spheroid formation.....	200
7.2.5	Potential for use in high-throughput technology	201

7.2.6	PSA EIA.....	203
7.2.7	GFP transfection	203
7.2.8	Cell viability following pPPE-CD/5-FC treatment.....	204
7.3	Discussion	213
Chapter 8	Discussion and conclusions	222
8.1	Improved efficacy of pPPE-CD under hypoxic conditions.....	223
8.2	A combined treatment strategy of pPPE-CD/5-FC and radiation therapy	224
8.3	Suitable controls for qRT-PCR analysis of hypoxic prostate cell samples...	226
8.3.1	Cell lines	227
8.3.2	Spheroids.....	228
8.4	Possible improvements of the PSA promoter	229
8.5	Possible improvements in CD efficiency	230
8.6	Potential of pPPE-CD/5-FC in the treatment of advanced and CRPC prostate cancer231	
8.7	Future development of GDEPT strategies	234
8.7.1	Improved delivery (transductional targeting)	234
8.7.2	Non-invasive imaging of transgene activity	237
8.8	Conclusion.....	240
Appendices.....		242
References.....		257

Tables

Table 1.1: GDEPT trials for the treatment of prostate cancer. Summary of clinical trials that used GDEPT to treat localised, locally recurrent and metastatic prostate cancer, including the toxicity and the outcome of the therapy..... 36

Table 2.1: Composition of standards for Bradford Assay 53

Table 2.2: Reagents in resolving gel..... 54

Table 2.3: Antibodies used in Western Blotting..... 55

Table 2.4: Gene Expression Assays..... 59

Table 3.1: Genes tested for suitability as endogenous control genes for qRT-PCR..... 75

Table 3.2: Mean Ct values for endogenous control genes 76

Table 3.3: Endogenous control genes ranked by their stability in normoxic (21% oxygen) and hypoxic (0.5%) samples using Normfinder 77

Table 3.4: Assessment of performance of endogenous control genes using coefficient of variation analysis in DU145 cells. Values represent the mean of three independent replicates. 78

Figures

Figure 1.1: The zones of the prostate..... 3

Figure 1.2: Estimated age-standardised rates of prostate cancer per 100,000 people. 5

Figure 1.3: Structure of the AR gene and protein..... 8

Figure 1.4: HIF-1 α regulation under normoxic and hypoxic conditions..... 11

Figure 3.1: Variability of 16 endogenous control genes in prostate cancer cell lines 22Rv1 and LNCaP cultured in 21% or 0.5% oxygen. 78

Figure 3.2: A) Coefficient of variation of C_t values between aerobic (21% oxygen) and hypoxic (0.5%) treatment groups for four candidate endogenous control genes in three prostate cancer cell lines following longer hypoxic exposure (48 hours). B) Three additional prostate cell lines were tested after growth under aerobic or hypoxic conditions for 48 hours. 79

Figure 3.3: Relative quantification of *HIF-1A* mRNA. 80

Figure 3.4: Average *GSTP1* mRNA expression levels in tumour samples relative to matched normal tissue using four different endogenous control genes. 82

Figure 4.1: pPPE-CD vector showing location of PSA promoter and enhancer and CD open reading frame. Numbers represent the positions in the GenBank sequence. 90

Figure 4.2: Mechanism proposed by Mabeesh *et al.* for HIF-1 α upregulation in LNCaP cells in response to DHT stimulation..... 92

Figure 4.3: PSA expression following 72 hours of DHT treatment in LNCaP (A) and 22Rv1 (B)..... 97

Figure 4.4: PSA protein levels measured by PSA ELISA following DHT stimulation in LNCaP under aerobic and hypoxic conditions. 98

Figure 4.5: PSA protein levels measured by PSA ELISA following DHT stimulation in 22Rv1 under aerobic and hypoxic conditions..... 99

Figure 4.6: Relative quantification of *PSA* mRNA in aerobic (21% oxygen) and hypoxic (0.5%) LNCaP samples treated with increasing concentrations of DHT. 100

Figure 4.7: Relative quantification of *PSA* mRNA in aerobic (21% oxygen) and hypoxic (0.5%) 22Rv1 samples treated with increasing concentrations of DHT..... 100

Figure 4.8: A) Western blotting for cystosine deaminase protein expression in pPPE-CD transfected LNCaP cells 101

xviii

Figure 4.9: A) Cytosine Deaminase protein expression in pPPE-CD transfected 22Rv1 cells 102

Figure 4.10: HIF-1 α protein expression in 22Rv1 cells treated with cobalt chloride.. 103

Figure 4.11: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected LNCaP cells treated with cobalt chloride and DHT..... 103

Figure 4.12: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected 22Rv1 cells treated with cobalt chloride and DHT. 104

Figure 4.13: Relative quantification of *HIF-1A* mRNA levels in siRNA transfected samples..... 105

Figure 4.14: A) Western blotting for HIF-1 α protein expression in 22Rv1 cells treated with increasing concentrations of the HIF-1 α inhibitor..... 106

Figure 4.15: A) Western blotting for HIF-1 α protein expression in 22Rv1 cells treated with increasing concentrations of the HIF-1 α inhibitor..... 107

Figure 4.16: LNCaP cell count after treatment in air. 109

Figure 4.17: LNCaP cell count after treatment in air with 1000 nM DHT..... 109

Figure 4.18: LNCaP cell count after treatment in hypoxia..... 110

Figure 4.19: LNCaP cell count after treatment in hypoxia with 1000 nM DHT..... 110

Figure 4.20: 22Rv1 cell count after treatment in air..... 111

Figure 4.21: 22Rv1 cell count after treatment in air with 1000 nM DHT..... 112

Figure 4.22: 22Rv1 cell count after treatment in hypoxia. 113

Figure 4.23: 22Rv1 cell count after treatment in hypoxia with 1000 nM DHT. 113

Figure 4.24: 22Rv1 cell numbers for untransfected (dashed line) and pPPE-CD transfected (solid line) cells grown in air (blue) or hypoxia (red) with 1000 nM DHT and treated with various concentrations of 5-FC..... 114

Figure 4.25: Cell colony number following clonogenic assays of 22Rv1 grown in air with or without DHT and treated with 5-FC..... 115

Figure 4.26: Cell colony number following clonogenic assays of 22Rv1 grown in hypoxia with or without DHT and treated with 5-FC..... 116

Figure 5.1: PSA protein levels measured by PSA EIA following DHT stimulation with or without irradiation in LNCaP 128

Figure 5.2: PSA protein levels measured by PSA EIA following DHT stimulation with or without irradiation in 22Rv1.....	129
Figure 5.3: Relative quantification of <i>PSA</i> mRNA in aerobic and hypoxic LNCaP samples treated with various doses of radiation.	130
Figure 5.4: Relative quantification of <i>PSA</i> mRNA in aerobic and hypoxic 22Rv1 samples treated with various doses of radiation.	131
Figure 5.5: Cytosine deaminase protein expression in pPPE-CD transfected and untransfected LNCaP cells grown in air.	132
Figure 5.6: A) Cytosine deaminase protein expression in pPPE-CD transfected LNCaP treated with DHT and/or 2 Gy under hypoxic conditions.....	132
Figure 5.7: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected 22Rv1 cells grown in air.	133
Figure 5.8: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected 22Rv1 cells grown in hypoxia.....	133
Figure 5.9: LNCaP cell count after treatment in air and irradiation with 2 Gy.	135
Figure 5.10: LNCaP cell count after treatment in air with 1000 nM DHT and irradiation with 2 Gy.....	135
Figure 5.11: LNCaP cell count after treatment in hypoxia and irradiation with 2 Gy.	136
Figure 5.12: LNCaP cell count after treatment in hypoxia with 1000 nM DHT and irradiation with 2 Gy.....	136
Figure 5.13: 22Rv1 cell count after treatment in air and irradiation with 2 Gy.	137
Figure 5.14: 22Rv1 cell count after treatment in air with 1000 nM DHT and irradiation with 2 Gy.....	138
Figure 5.15: 22Rv1 cell count after treatment in hypoxia and irradiation with 2 Gy..	139
Figure 5.16: 22Rv1 cell count after treatment in hypoxia with 1000 nM DHT and irradiation with 2 Gy.....	139
Figure 5.17: Absorbance following MTT assay for pPPE-CD transfected 22Rv1 cells supplemented with DHT and treated with 1mM 5-FC with and without radiation.	140
Figure 5.18: Cell colony number following clonogenic assays of 22Rv1 grown in air with 2Gy radiation, with or without DHT and treated with 5-FC.	141

Figure 5.19: Cell colony number following clonogenic assays of 22Rv1 grown in hypoxia with 2Gy radiation, with or without DHT and treated with 5-FC. 141

Figure 5.20: A) Androgen receptor protein expression in LNCaP with and without radiation treatment. 142

Figure 5.21: A) Androgen receptor protein expression in 22Rv1 cells exposed to radiation and DHT treatment. 143

Figure 5.22: Relative quantification of *AR* mRNA in aerobic and hypoxic LNCaP samples treated with various doses of radiation. 144

Figure 5.23: Relative quantification of *AR* mRNA in aerobic and hypoxic 22Rv1 samples treated with various doses of radiation. 144

Figure 5.24: A) NF-κB protein expression in 22Rv1 cells exposed to radiation and DHT treatment under aerobic conditions..... 145

Figure 5.25: A) NF-κB protein expression in 22Rv1 cells exposed to radiation and DHT treatment under hypoxic conditions..... 146

Figure 6.1: pcDNA3.1-AR following digestion with the restriction enzymes BglII and HindIII, as well as uncut plasmid as a control..... 159

Figure 6.2: Cell survival of 22Rv1 following Geneticin treatment. 160

Figure 6.3: A) AR expression levels measured by Western Blotting. 161

Figure 6.4: A) AR protein expression in response to DHT treatment. 162

Figure 6.5: Quantification of AR mRNA levels relative to 22Rv1 untreated. 163

Figure 6.6: PSA protein levels following DHT stimulation in 22Rv1 under aerobic (A) and hypoxic conditions (B)..... 164

Figure 6.7: Quantification of PSA mRNA levels relative to 22Rv1 untreated under aerobic conditions. 165

Figure 6.8: Quantification of PSA mRNA levels relative to 22Rv1 untreated under hypoxic conditions. 165

Figure 6.9: Relative quantification of FKBP51 expression in 22Rv1 and 22Rv1-ARhi under aerobic (A) and hypoxic (B) conditions. 166

Figure 6.10: Cell count following treatment with various concentrations of DHT for seven days. 167

Figure 6.11: Absorbance levels after MTT assay of cells grown for 3 days with various concentrations of DHT under aerobic conditions. 168

Figure 6.12: Absorbance levels after MTT assay of cells grown for 3 days with various concentrations of DHT under hypoxic conditions. 168

Figure 6.13: A) CD expression in pPPE-CD transfected 22Rv1 and 22Rv1-ARhi cells under aerobic and hypoxic conditions. 169

Figure 6.14: A) CD expression in pPPE-CD transfected 22Rv1 and 22Rv1-ARhi cells grown under aerobic conditions with DHT and/or radiation treatment. 170

Figure 6.15: Cell number following bicalutamide treatment under aerobic conditions. 171

Figure 6.16: Cell number following bicalutamide treatment under hypoxic conditions. 172

Figure 6.17: Cell colony number following bicalutamide treatment. 173

Figure 6.18: A) Nuclear protein expression levels of AR in LNCaP, 22Rv1 and 22Rv1-ARhi samples from untreated cells or cells treated with 0.1 nM DHT..... 174

Figure 6.19: A) Nuclear protein expression levels of AR in LNCaP, 22Rv1 and 22Rv1-ARhi cell samples treated with 10 nM or 1000 nM DHT..... 175

Figure 6.20: A) AR protein expression in 22Rv1 cells treated with the calpain activator ionomycin 176

Figure 6.21: A) AR protein expression in 22Rv1-ARhi cells treated with the calpain activator ionomycin 177

Figure 6.22: AR protein expression in 22Rv1 cells treated with the calpain inhibitor calpastatin 178

Figure 6.23: AR protein expression in 22Rv1-ARhi cells treated with the calpain inhibitor calpastatin..... 179

Figure 6.24: Secreted PSA levels following DHT (1000 nM) stimulation with calpastatin or ionomycin treatment in 22Rv1 and 22Rv1-ARhi..... 181

Figure 6.25: Secreted PSA levels following DHT (1000 nM) stimulation with calpastatin or ionomycin treatment in LNCaP 182

Figure 7.1: 22Rv1 cells seeded in HC media demonstrating the combination of spheroid formation and adherence of cells. 197

Figure 7.2: Left: 22Rv1 cells seeded in HC media using a ‘layering’ technique to reduce cell adherence. Right: a well in which the cells were mixed upon addition to HC media..... 198

Figure 7.3: 22Rv1 cells seeded in HC media in ‘Hydrocell’ 96-well plates. 198

Figure 7.4: GFP-transfected 22Rv1 spheroids transferred from a 24-well to 96-well plate..... 199

Figure 7.5: A representative spheroid stained with Hoechst 200

Figure 7.6: Representative spheroids stained with Hoechst and PI..... 200

Figure 7.7: 22Rv1 cells seeded in HC media in a 96-well plate and imaged at 4 × magnification over 5 days..... 201

Figure 7.8: Cell number per well after automatic liquid dispensing at different time points using a cell suspension of HC media and normal DMEM media. 202

Figure 7.9: 22Rv1 cells in a well of a nanowell slide, imaged over the course of 3 days 202

Figure 7.10: PSA protein levels following DHT stimulation in 22Rv1 adherent cells and spheroids. 203

Figure 7.11: GFP-transfected 22Rv1 cells grown as adherent cells (A & B) and spheroids (C & D) 5 days after seeding. 204

Figure 7.12: 22Rv1 cell count after treatment in air with and without DHT (1000 nM). 205

Figure 7.13: 22Rv1 cell count after treatment in air with radiation and with and without DHT (1000 nM). 206

Figure 7.14: pPPE-CD transfected 22Rv1 cell number after treatment in air with radiation and/or 5-FC..... 207

Figure 7.15: 22Rv1 cell count after treatment in hypoxia with and without DHT..... 208

Figure 7.16: 22Rv1 cell count after treatment in hypoxia with radiation and with and without DHT. 208

Figure 7.17: 22Rv1 spheroid size after treatment in air with and without DHT. 210

Figure 7.18: 22Rv1 spheroid size after treatment in air with radiation and with and without DHT. 210

Figure 7.19: 22Rv1 spheroid size after treatment in hypoxia with and without DHT. 211

Figure 7.20: 22Rv1 spheroid size after treatment in hypoxia with radiation and with and without DHT. 211

Figure 7.21: pPPE-CD transfected 22Rv1 spheroid size after treatment in air with radiation and/or 5-FC. 212

Figure 8.1: Transductional and transcriptional targeting of GDEPT..... 235

Abbreviations

°C	Degrees Celsius
5-FC	5-fluorocytosine
Ad	Adenovirus
APS	Ammonium persulfate
AR	Androgen receptor
ARE	Androgen response element
BSA	Bovine serum albumin
CAR	Coxsackie B virus and adenovirus receptor
CD	Cytosine deaminase
CD-FBS	Charcoal dextran stripped fetal bovine serum
CMV	Cytomegalovirus
CRPC	Castration resistant prostate cancer
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNase	deoxyribonuclease
EBRT	External beam radiotherapy
EIA	Enzyme immunoassay
FBS	Fetal bovine serum
GCV	Ganciclovir
GFP	Green fluorescent protein

HRE	Hypoxia response element
HRP	Horseradish peroxidase
HSVtk	Herpes simplex virus thymidine kinase
IMRT	Intensity modulated radiotherapy
kDa	Kilodaltons
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
PI	Propidium iodide
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
qRT-PCR	Quantitative reverse transcriptase PCR
rpm	Revolutions per minute
TEMED	N,N,N',N'-tetramethylethylenediamine
UPRT	uracil phosphoribosyltransferase

Chapter 1

Introduction

Chapter 1 Introduction

1.1 The prostate gland

1.1.1 Structure

The anatomy of the prostate gland is most commonly described as a series of zones (Figure 1.1), as first put forward by McNeal in 1968.¹ The transition zone makes up 5-10% of the mass of the gland in young adult males and consists of two symmetrical lobes that surround the urethra.^{2, 3} The central zone takes up about 25% of the prostate and the largest zone is the peripheral zone at 70 – 75%, which surrounds the central zone.²⁻⁴ The anterior fibromuscular stroma consists of striated muscle at the top, which mixes with the gland itself, and smooth muscle at the bottom, which joins with fibres from the bladder neck.^{2, 4}

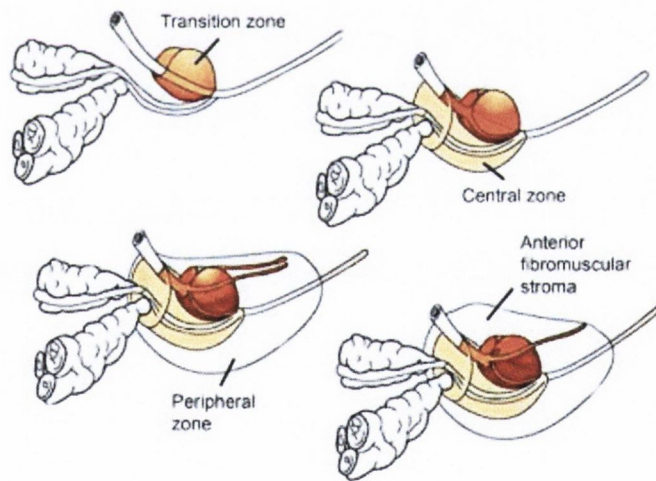


Figure 1.1: The zones of the prostate.²

1.1.2 Function

The prostate gland produces prostatic fluid which moves into the urethra during ejaculation to form part of the seminal fluid.^{5, 6} This fluid contains a number of substances, such as citric acid, zinc, polyamines and the proteins prostate specific antigen (PSA) and prostatic acid phosphatase.⁵ PSA is a serine protease made by the

prostate epithelial cells and acts on seminal vesicle proteins in the lumen of the prostate to make the semen liquid.³ During ejaculation, the smooth muscles of the prostate contract to help push the semen along the urethra.^{5, 6}

1.2 Prostate cancer

1.2.1 Epidemiology

Worldwide, prostate cancer is the second most common cancer among men, with 903,000 new cases diagnosed in 2008 and 258,000 deaths.⁷ The incidence is highest in Australia, New Zealand, Western and Northern Europe and North America, with the rates varying by more than 25-fold between different parts of the world.⁷ Rates are lowest in Asia and Northern Africa (Figure 1.2). In Ireland, prostate cancer is the most frequently diagnosed form of cancer for men, with a rate of 140.1 per 100,000 per year for the period 2007-2009. The mortality rate was 26.5 per 100,000.⁸ Multiple elements are likely to contribute to the disparity between the rates of prostate cancer in different countries. One contributing factor is country-to-country variation in the extent of screening for the biomarker PSA, although there were differences between the rates of prostate cancer incidence before PSA testing was introduced so it is not the only cause.⁹ Differences in genetic susceptibility and environmental influences are likely to be involved. In one study, the rate of prostate cancer among Japanese men living in North America was found to be five times higher than those living in Japan, although only half that of European Americans living in the same area.¹⁰ Such results suggest that both genetic and environmental factors affect prostate cancer rates.

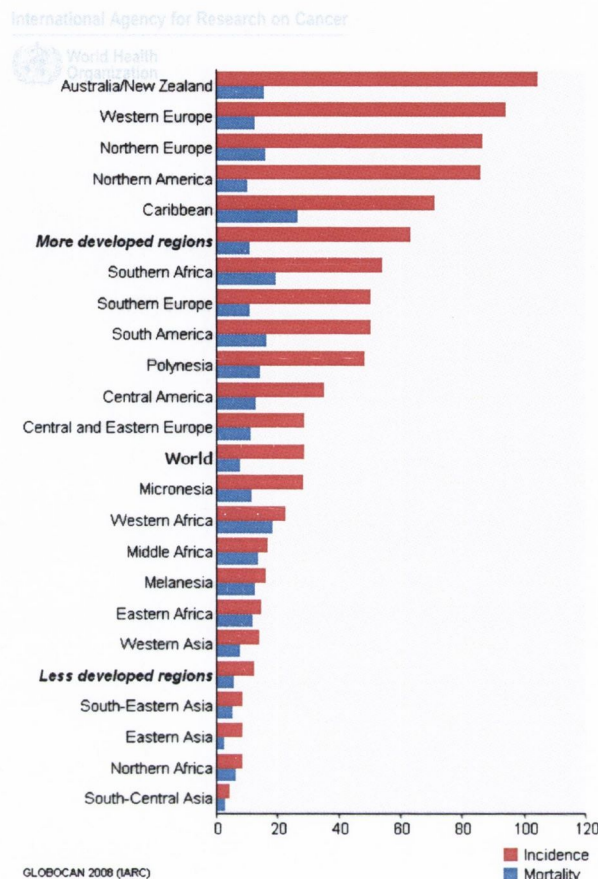


Figure 1.2: Estimated age-standardised rates of prostate cancer per 100,000 people.⁷

The occurrence of prostate cancer in men younger than fifty years is rare with the levels increasing with age. In North America, between 2003 and 2007, 8.9% of those diagnosed with prostate cancer were aged 45-54; 29.9% were aged 55-64, 35.3% were aged 65-74, 20.7% were 75-84 and 4.6% were 85 or older.¹¹

1.2.2 Symptoms

The majority of patients diagnosed with prostate cancer are asymptomatic,¹² however there are some symptoms associated with the disease. For example, in cases of locally advanced prostate cancer, movement of the tumour into the surrounding bodies may lead to impotence, haematuria and/or urinary obstruction.^{3, 12} If the cancer is metastatic and present in the skeleton, symptoms may include pain in the lower back and

pathological fractures. Lymph node metastasis may cause enlargement of lymph nodes and lymphoedema.³

1.2.3 Progression

Prostate cancer is an adenocarcinoma, a cancer of the epithelium. Prostatic intraepithelial neoplasia (PIN) is the precursor to prostate cancer, whereby proliferation of the epithelial cells results in a layer of crowded irregular cells. However, PIN differs from adenocarcinoma as there is still an intact basal cell layer.¹³ It is divided into low-grade PIN (LGPIN) and high-grade PIN (HGPIN), HGPIN being the clinically relevant form. This supercedes previous classification into PIN 1, 2 or 3, based on the degree of cell changes, with LGPIN replacing PIN 1 and HGPIN replacing PIN 2 and 3.¹⁴ It is non-invasive and may never develop into cancer, or develop so slowly that it never affects the patient. However, 80-90% of radical prostatectomy specimens will contain HGPIN, sometimes when it has directly transitioned into invasive disease.¹⁵

Progression to adenocarcinoma is characterized by breakdown of the basal cell layer, cell detachment and invasion.¹⁶ This requires the abnormal expression of adhesion molecules and anchorage independence, the ability to grow when not attached to the basal membrane. Once the disease has become invasive, the degree of aggressiveness and progression will depend on a variety of genetic factors. At diagnosis, the cancer will be given a Gleason grade as a way of categorizing the degree of abnormality of the cell structure and evaluating prognosis.¹⁷

1.2.4 Diagnosis

The optimal means of testing for prostate cancer involves combining a digital rectal examination (DRE) with a measurement of PSA levels.^{12, 18} A fraction of PSA will enter the blood stream, where it forms a complex with alpha-1-antichymotrypsin (ACT) or, to a lesser extent, alpha-2-macroglobulin or alpha-1-antitrypsin. A small amount will remain free.^{3, 19} The levels of PSA in the blood increase with the presence of a tumour due to the disruption of the prostate structure and an increase in the number of cells.¹⁹ The subsequent PSA value, however, is neither sensitive nor specific enough to give a definite diagnosis.²⁰ Men whose levels of PSA exceed a given threshold

(>4ng/ml) will be subjected to an ultrasound-guided prostate biopsy to confirm the diagnosis.²¹ Up to one third of these individuals are found to be cancer free. Furthermore, it is estimated that approximately 25% of biopsies give a false negative result.²⁰ It is thought that elevated PSA levels may in fact be a better marker of benign prostate hypertrophy (BPH) than prostate cancer.

1.3 The Androgen Receptor

The androgen receptor (AR) is an important transcription factor, controlling the expression of a large number of genes that are key during development, as well as the maintenance of apoptosis, proliferation, differentiation and metabolism.²² AR also plays a central part in the development and progression of prostate cancer and controls expression of the biomarker PSA.

AR is a member of the steroid and nuclear receptor superfamily that also includes mineralocorticoid, glucocorticoid, oestrogen and progesterone. It consists of four domains: the amino-terminal domain (NTD), DNA-binding domain (DBD), hinge region and ligand binding domain (LBD). The sequence of the NTD is highly variable. It contains three repeat regions and the length of these determines the stability of the interaction between the NTD and LBD. This is an important step in the activation of the AR so variation in the NTD repeat regions affects AR activity.^{23, 24} The NTD is transcriptional activation function-1 (AF1) and contains transcriptional activation unit-1 (TAU-1) and TAU-5. In contrast to the NTD, the DBD has a highly conserved sequence and is responsible for the recognition of and binding to androgen response elements (AREs) in the promoters and enhancers of specific genes. It contains two zinc fingers, the first of which binds to the DNA major groove in specific regulatory regions and the second binds to the DBD of another AR to allow homodimer formation in the nucleus.²⁵ The short, 50 amino acid hinge domain links the DBD and LBD and contains part of the nuclear localisation signal, the other part of which is in the DBD.²⁶

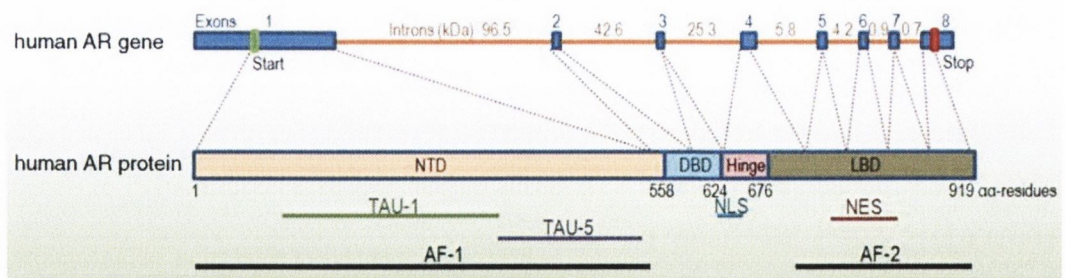


Figure 1.3: Structure of the AR gene and protein. The protein is made up of four different domains: amino-terminal domain (NTD), DNA-binding domain (DBD), hinge region and ligand binding domain (LBD).²⁷

The activation of the AR is controlled at the LBD through binding of the androgens testosterone or dihydrotestosterone (DHT). In the absence of androgen, AR is located in the cytoplasm and is associated with a number of chaperone proteins, including heat shock proteins. When ligands bind to the LBD, there is a conformational change resulting from the the formation of the AF2 from helices 2,4 and 12 of the LBD and the interaction of the LBD with the NTD stabilising the AR.²⁸ Helix 12 of the LBD moves across the ligand binding pocket to prevent dissociation.^{29, 30} The chaperone proteins dissociate and various co-regulators bind to the nuclear localisation signal, followed by the shuttling of the AR into the nucleus. There, AR binds to AREs in androgen-responsive genes, which results in the binding of the transcriptional machinery as well as specific co-regulators and histone deacetyltransferase enzymes and subsequent transcription of the gene.^{27, 31}

A truncated form of the AR was first identified in human genital skin fibroblasts³² and has since been discovered in a range of human tissues.³³ 22Rv1, a primary prostate tumour cell line, contain two forms of the AR protein, differing in length; the full-length AR (fAR) is 114 kDa and the truncated AR is 75-80 kDa (trAR). The COOH-terminally truncated version of the AR in 22Rv1, which lacks the ligand binding domain, was identified by Tepper *et al.*³⁴ In the presence of androgens, fAR and trAR could be detected in both cytoplasmic and nuclear extracts. In the absence of androgen, the levels of trAR in the nucleus were maintained but were reduced for fAR. In this

androgen depleted media, the protein expression of fAR increased with addition of increasing concentration of the artificial androgen mibolerone. trAR was demonstrated to contain a functional DNA-binding domain and bind AREs. Tepper *et al.* identified a tandem duplication of exon 3 which increases the size of fAR in 22Rv1 by 5 kDa. To elucidate the importance of this mutation, exon 3 in a wild type AR expression construct was replaced with the sequence from 22Rv1 and then transfected into 293T (embryonic kidney) cells. trAR as well as fAR was detected in these cells but only fAR was expressed in 293T cells transfected with the original wild type AR expression construct. In PC3 cells (derived from bone metastases of prostate carcinoma) transfected with both the AR expression constructs and an AR-responsive luciferase reporter plasmid and treated with 1 nM DHT, the exon 3 mutation resulted in a 33% lower induction of luciferase activity compared to WT AR.

Because the AR is such an important transcription factor, its dysregulation can lead to the initiation and progression of prostate cancer and it is thought to have a role in all stages of the disease. As mentioned, the sequence of the NTD is highly variable and the repeat length of the CAG trinucleotide may affect risk of BPH (≤ 19 repeats) or prostate cancer (≤ 18 repeats). A shorter CAG repeat sequence results in increased responsiveness of the AR to androgen. However examination of the association between heritable CAG repeat length and risk of prostate cancer has resulted in mixed results.^{35, 36} Somatic shortening of the CAG sequence, on the other hand, has been associated with prostate cancer; cancerous tissue has shown genetic heterogeneity and significantly shorter CAG sequences compared to the adjacent benign tissue.³⁷

Patients who are treated with androgen deprivation therapy will inevitably proceed to having castration resistant prostate cancer (CRPC) at a median of 2-3 years after initiation of treatment with average survival of a further 16-18 months.³⁸ This progression is being studied and a number of different mechanisms have been suggested based on current evidence, which can be broadly divided into two groups, AR-dependent and AR-independent.³⁹ The possible AR-dependent mechanisms are: 1) amplification of AR leading to overexpression 2) mutations that result in AR stimulation by non-androgenic steroids and anti-androgens 3) changes in the levels of

AR co-regulators 4) changes in the levels of steroidogenic enzymes and 5) up-regulation of transcription factors that regulate AR through cross-talk. The AR-independent mechanisms involve prostate stem cell regeneration or alternative pathways to AR regulation^{39, 40}

1.4 Hypoxia

Hypoxia, or abnormally low levels of oxygen, induces numerous stress related signalling pathways in the cell. This is mediated by an inducible transcription factor, hypoxia inducible factor 1 (HIF-1). HIF-1 was first described when it was found that, during hypoxia, it activated erythropoietin expression, a hormone that regulates erythrocyte levels to control oxygen levels in tissue.^{41, 42} Today, many genes are known to be activated by HIF-1 and these genes are involved in a diverse range of processes including cell survival, oxygen transport, apoptosis and angiogenesis.^{41, 43}

HIF-1 binds to enhancers known as hypoxia response elements (HREs) found in numerous genes. HREs contain the conserved core sequence 5'-(A/G)CGT(G/C)(G/C)-3'. Activation of the HREs initiates the cellular response to hypoxia, leading to increased oxygen delivery and aiding in survival.^{44, 45} HIF-1 is a heterodimer made up of two subunits, HIF-1 α and HIF-1 β , from a protein family that contain basic helix-loop-helix Per/Arnt/Sim domains (bHLH-PAS).⁴⁶ Both proteins are constitutively expressed. However, although HIF-1 β is found in all cell types regardless of oxygen concentration, the presence of HIF-1 α is tightly controlled and is dependent on oxygen levels.^{41, 47} Under normoxic conditions, HIF-1 α is hydroxylated, a process catalyzed by the prolyl hydroxylases (PHDs) of which there are four isoforms (PHD1, PHD2, PHD3 and PHD4). Once hydroxylated, HIF-1 α is recognised by the prolyl hydroxylase von Hippel-Lindau protein (pVHL), resulting in polyubiquitination of HIF-1 α (Figure 1.4). As well as that, HIF-1 α undergoes lysine acetylation in a normoxic environment. Both of these processes target HIF-1 α for degradation by the proteasome.⁴⁶ Therefore, when normoxic conditions are present in the cell, HIF-1 α has a half life of less than 5 minutes.⁴¹ Degradation of HIF-1 is prevented under hypoxic conditions, however, as pVHL is no longer active and acetylation is downregulated.⁴¹ HIF-1 α translocates to the nucleus and binds to HIF-1 β forming the DNA binding complex HIF-1 which is

associated with co-activator proteins p300/CBP (CREB-binding protein).⁴⁶ This results in the upregulation of target genes associated with proliferation, angiogenesis and glucose metabolism, for example.⁴⁸

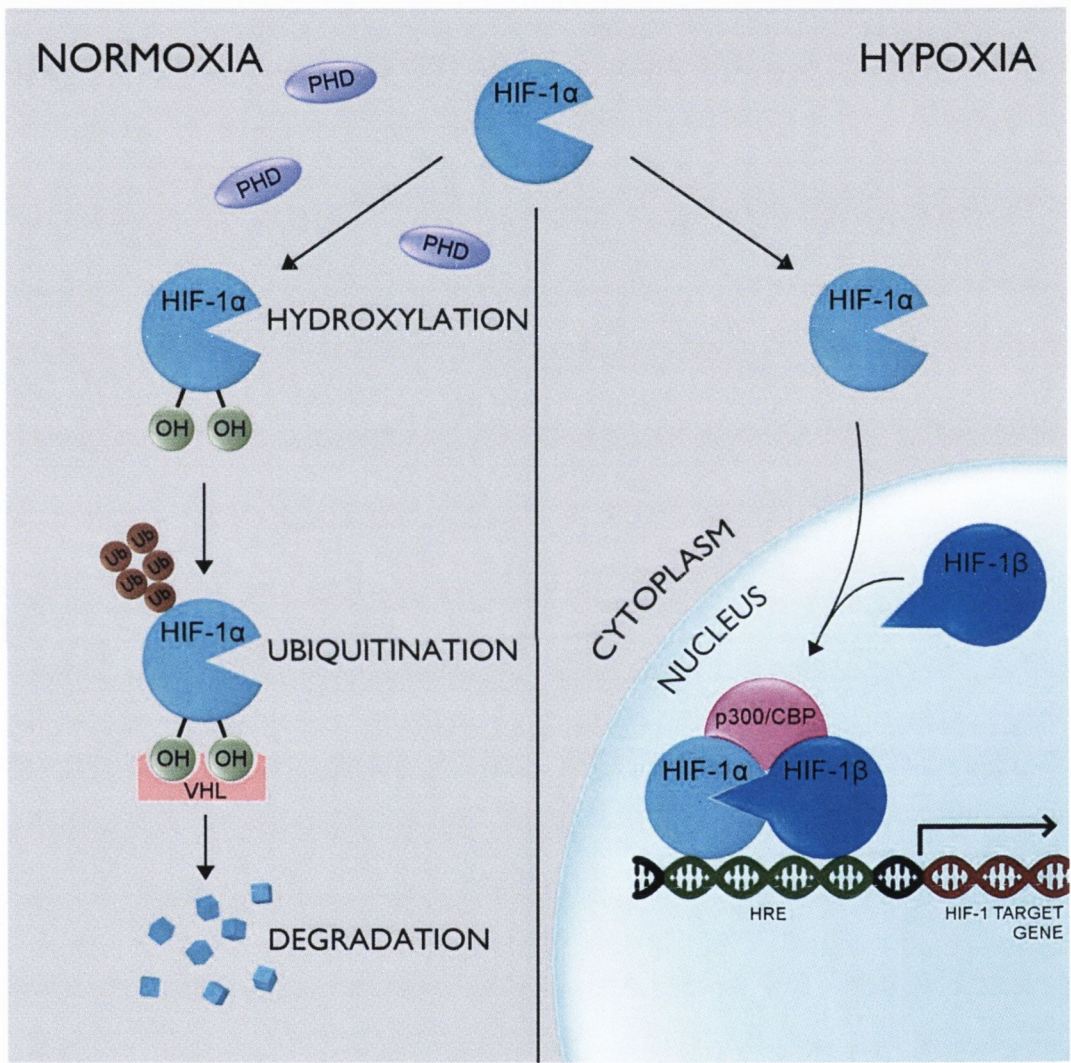


Figure 1.4: HIF-1α regulation under normoxic and hypoxic conditions. PHD, prolyl hydroxylase; OH, hydroxyl group; Ub, ubiquitin; VHL, von Hippel-Lindau protein; HRE, hypoxia response element.

Aberrant gene expression is a hallmark of human cancer. Over the past decade, qRT-PCR has become the gold-standard technique for quantifying gene expression changes. This method commonly employs a housekeeping gene (HKG) as an endogenous

control to normalise results, the choice of which is critical for accurate data interpretation. Tissue type, treatment effects and oxygen levels are just some examples of factors that can influence gene expression levels, as well as those of the putative HKG.

It is estimated that up to 5% of all genes are regulated by HIF-1 in response to hypoxia in one or more cell types.⁴³ There is great heterogeneity in the genes that are regulated by HIF-1 between different cell lines, even those that originate from the same organ or tumour site. This is evidence that growth in a hypoxic environment could affect the expression of potential endogenous control genes for qRT-PCR and that merely testing one or two cell lines from a panel is not sufficient to find a suitable HKG. One aim of this research project was to determine the suitability of commonly used HKGs for gene expression analysis in prostate cancer and to compare their performance under aerobic and hypoxic conditions.

HIF-1 over-expression occurs in prostate cancer, as well as many other cancer types and is associated with cancer progression.^{44, 45} This has been demonstrated through measurement of hypoxia markers using immunomolecular imaging and physical measurements.⁴⁵ The rapid growth of tumours results in a disorganised vascular network, which cannot keep pace with the oxygen demand. Blood flow may be impaired by malformations of the blood vessels. Oxygen cannot reach some parts of the tumour, causing them to be hypoxic as a consequence⁴⁹⁻⁵¹. Hypoxia has been associated with tumour aggressiveness and metastasis due to the up-regulation of a host of genes involved in angiogenesis and glycolysis. Consequently, hypoxia is associated with poor prognosis in several cancer types, including prostate^{44, 51}. It has also been associated with resistance to both chemotherapy and radiation therapy.⁴⁵ The presence of oxygen is important for successful cell kill by ionizing radiation: initially, the ionizations produce a free radical on the DNA. This causes damage to the DNA and if the free radicals are oxidized this potentiates the damage. If oxygen is not present, the free radicals are reduced by hydrogen and the DNA damage can be repaired.⁵² Therefore, under hypoxic conditions, when oxygen levels are low, ionizing radiation is less effective at killing cells. A study by Nahum *et al.* using a 'tumour local control

probability model' indicated that the presence of hypoxic cells was a key factor in determining success of radiation therapy.⁵³

One focus of this research is the study of cross-talk in the transcriptional regulation of AR and HIF-1 activated genes. It is now known that growth of cells under hypoxic conditions can further upregulate androgen stimulated genes and in a synergistic manner the expression of some hypoxia responsive genes is increased with the addition of androgens. This is an important factor in prostate cancer progression and resistance to treatment. Crosstalk will be discussed in further detail in section 4.1.2.

1.5 Current treatments for prostate cancer

1.5.1 Management of localised prostate cancer

Current clinical practice utilises two radical forms of therapy for organ-confined prostate cancer with curative intent, the option chosen depending on the tumour stage and Gleason grade of the disease, as well as PSA levels at detection.⁵⁴ These treatments are surgery, in the form of retropubic radical prostatectomy and radical radiation therapy.

Radical prostatectomy (RP) involves the surgical removal of the prostate along with the seminal vessicals and occasionally the pelvic lymph nodes, while simultaneously minimising damage to the surrounding nerves to prevent incontinence and erectile dysfunction.⁵⁵ RP can take the form of open surgery, retropubic or perineal, or more recently laproscopic or robotic surgery. The latter are less invasive but are also harder to perform with a steep learning curve.⁵⁵ Short term risks include pulmonary embolism, deep vein thrombosis and damage to the rectum. In the long term, there is a low risk of incontinence and a higher risk of loss of sexual function.⁵⁶

External beam radiotherapy (EBRT) involves the administration of high-energy photon beams to the prostate every day over a number of weeks.^{57, 58} There have been significant advances in this therapy in recent times. Progress in technology has allowed for better tumour targeting, as well as escalated doses of radiation to cell kill.^{59, 60} Traditional radiotherapy was restricted to doses of 65-70 Gy due to the risk to normal

tissue. However this was shown to be insufficient for the eradication of localised tumours.⁶¹⁻⁶⁴ Nowadays, this two-dimensional method has been widely replaced with three-dimensional conformal radiotherapy (3D-CRT) in which the shape and volume of the tumour are defined, allowing the radiation beams to be precisely targeted.^{60, 65} This has allowed for an increase in the radiation dose to 72-76 Gy, without a concurrent increase in toxicity.^{66, 67} Intensity modulated radiotherapy (IMRT) represents further progression in the field; this allows for manipulation of radiation dose across the beam giving even greater command of the tissues targeted.⁶⁰

The acute side-effects of EBRT mainly result from damage to the bladder and rectum. These are seen in around half of men but are normally treatable and last only a few weeks.⁵⁷ There is a relatively low level of clinically relevant side-effects, less than ten percent when conformal radiotherapy is used, including hematochezia, hematuria and rectal urgency as well as erectile dysfunction.^{57, 58}

Brachytherapy is a form of radiation therapy that involves the insertion of sources of radiation into the tumour site.⁵⁷ High doses of radiation can be administered to the prostate but these do not travel as far as the nearby healthy tissues. There are two forms of brachytherapy: low-dose-rate (LDR) and high-dose-rate (HDR). LDR brachytherapy is more commonly used for the treatment of prostate cancer. It involves the placement of 'seeds' in the tumour that contain radioactive isotopes, usually iodine-125 or palladium-103, with doses of 145 and 120 Gy respectively.^{57, 68} In the case of HDR brachytherapy, catheters are put into the prostate and a single radioactive source is then placed at different locations inside the catheter for a number of minutes.⁵⁷ Compared to EBRT, the risk of a urinary related side-effect is higher but for rectal related side-effects the risk is lower. The chances of developing erectile dysfunction are about the same.⁵⁸

The technological advances in radiation therapy have led to better treatment results. However the higher levels of tumour destruction achieved by increasing radiation levels must be balanced with the risk of concurrent damage to normal tissue. In addition, subpopulations of clonogenic tumour cells may be radioresistant despite high dose

levels, leading to local recurrence and/or formation of metastases in a proportion of the patients.⁵⁹ As a consequence there is a clear requirement for the exploration of additional avenues to further improve the efficacy of radiation therapy.

For those with low risk disease, some suggest the best option is active surveillance with the idea that a “wait-and-see” approach would avoid or defer the negative side effects of treatment in these men⁶⁹. However, it would not be suitable to merely wait until the development of metastatic disease before intervening – instead close monitoring of biochemical changes is necessary, as well as selection of suitable candidates, for example those with low-risk disease (Gleason Score ≤ 6 , T1c-T2a, PSA < 10).⁵⁴ The Gleason Score runs from 2 (low-risk) to 10 (high-risk).

There is currently a dearth of research comparing the outcomes of these alternative treatments (RT, RP and active surveillance) in randomised clinical trial, meaning it is unclear which is the most effective, getting the best balance between control of the cancer and adverse effects. This problem is compounded by the difficulty in directly comparing the various methods due to issues such as patient selection bias – older patients tend to be treated with RT rather than the more invasive RP⁷⁰. Other complications include the rapid progress in RT techniques; the possible use of adjuvant, neoadjuvant or both in combination with RT; as well as variations in the definition of recurrence after RP and RT⁵⁵.

Most information comes from retrospective studies to compare the treatments. Results from these have been mixed: Tewari *et al.* looked at survival in patients with clinically localised prostate cancer who were treated with either watchful waiting, RT or RP.⁷¹ This study involved 3159 men of 75 years or younger. Following adjustment for age, race, tumour grade, comorbid disease, income status and year of diagnosis, it was found that the overall survival rate at 15 years was 35% for watchful-waiting, 50% for RT and 65% for RP. Prostate cancer-specific survival rates were 79%, 87% and 92% respectively. The results indicated patients treated with definitive therapy had increased chance of survival compared to conservative treatment.

There has been only one randomised prospective trial which has compared RP with watchful waiting as methods of early prostate cancer management and was initiated by the Scandinavian Prostate Cancer Group.⁷² Between 1989 and 1999, 695 men aged 75 and younger were randomly allocated treatment by RP (347) or watchful waiting (348). Results were published in 2005, following a median of 8.2 years of follow-up and showed RP improved prostate cancer survival in comparison to the watchful waiting approach: the relative reduction in risk of prostate cancer mortality was 44%, in risk of metastases was 40% and in overall mortality was 26%.⁷³ In the most recently published data, with a median follow-up of 10.8 years, the relative reduction in risk of prostate cancer mortality was 35%, in risk of metastases was 35% and in overall mortality was 18% in favour of RP.⁷⁴

The results of this study indicate treatment with RP, as opposed to watchful waiting, increases chance of survival for patients with localised prostate cancer. However, it is important to note, as the researchers themselves do, that the majority of the men involved in the study were not diagnosed using PSA screening. Therefore it is unclear if these results are applicable to modern prostate cancer patients who are mostly diagnosed early, due to advances in PSA testing, but who may be asymptomatic and have a low chance of actually dying from prostate cancer.⁷⁴

1.5.2 Management of local failure following therapy

A subset of men treated with radiation therapy will experience treatment failure and recurrence of the disease. Once biochemical failure has been established, a non-curative follow-up treatment option is salvage androgen suppression therapy. This is achieved in most cases through the administration of luteinizing hormone-releasing hormone (LHRH) agonists, possibly combined with an anti-androgen.⁷⁵ Side effects resulting from androgen deprivation therapy include hot flushes, lack of libido, gynecomastia, cognitive dysfunction and depression and over time patients can develop osteoporosis and anaemia and longer term risk of accelerated ischaemic heart disease.⁷⁶

Curative treatment strategies used after radiation therapy are uncommon but include salvage radical prostatectomy. This treatment results in a cancer-specific survival rate

at 10 years of 65-73% in highly selected patients but the side effects are significant, due in part to the difficult nature of the surgery following radiation-induced damage to the pelvic tissues.⁷⁷⁻⁸¹ These include erectile dysfunction and incontinence.^{77, 78} Salvage cryoablation is an alternative investigational method which allows for destruction of locally recurrent tumour. Of note, the patients treated in this way frequently suffer side effects. For example, Bales *et al.* were able to achieve negative biopsy in 86% of their patients at 3 months post-cryoablation but 95% of the patients suffered from incontinence.⁸² However, the technique has been developed and improved since then, moving away from using liquid nitrogen cryoprobe to argon/helium gas.⁸³ Ghafar *et al.* attained biochemical disease-free survival in 86% and 74% of patients at one and two years respectively using argon gas cryoablation and the level of incontinence was 7.9%.⁸⁴

In patients where the tumour remains localised another option is salvage brachytherapy which involves the administration of further higher doses of radiation through the use of radioactive seeds or catheters.⁸⁵ Recently, four year biochemical disease-free survival rates of 70% and 75% have been obtained using this technique, with the most common side effects being gastrointestinal and genitourinary toxicity.^{86, 87} Finally, some groups have used high intensity focused ultrasound (HIFU) as a method of salvage therapy following RT.⁸⁸⁻⁹⁰ Ultrasound energy is targeted to a specific area causing the temperature to increase to more than 60°C, resulting in cell death.⁸⁸ There are limited data on the efficacy of HIFU in the context of salvage therapy. In the largest to date, 3 year progression-free survival rates were 53%, 42% and 25% for low, intermediate and high-risk patients respectively, although there was a high level (49.5%) of urinary incontinence in the patient cohort.⁸⁹

1.5.3 Management of metastatic prostate cancer

In a proportion of men, either due to late diagnosis or treatment failure, prostate cancer will become androgen independent and metastasise. Most tumours initially metastasise to bone, but distant lymph nodes, the lungs and the liver may become involved. Osseous metastases are found in about 90% of those with advanced disease.^{91, 92} Currently, docetaxel-based chemotherapy for metastatic disease improves bone pain

and quality of life.⁹³ However its use is predominantly palliative, with a modest gain in long-term survival.⁹⁴

1.5.4 Neoadjuvant/adjvant therapy

In an attempt to improve the efficacy of radical prostatectomy and radiation therapy and to reduce the risk of recurrence of the disease, these standard therapies have been combined with neoadjuvant therapy (given prior to the main treatment) or adjuvant therapy (given after). In the case of hormonal neoadjuvant and adjuvant therapy, activation of the androgen receptor is targeted - it is blocked by anti-androgens (e.g. bicalcutamide, nilutamide) or the amount of androgen is reduced using leutinizing hormone releasing hormone (LHRH) agonists (e.g. goserelin, buserelin). This is known as androgen deprivation therapy (ADT).^{95, 96} ADT is administered in the hope that it will reduce the size of the tumour, making surgical resection easier or, in the case of RT, decrease the target area, therefore decreasing the amount of healthy tissue in contact with high dose radiation.^{69, 95, 96}

The results of research into the benefits of combining hormonal therapy with RP or RT are heterogeneous and comparison is made difficult by the variation in many of the study parameters, such as the specific nature of the hormone treatments and the duration and timing of the treatment. There is also variation in the criteria used for patient selection regarding disease stage and whether restrictions were placed on upper PSA levels. The classification of biochemical disease recurrence differs, as does the staging system used.⁹⁵

Meta-analyses, as well as systematic reviews of trials of adjuvant/neo-adjuvant therapy, have been published for localised and locally advanced prostate cancer. The most recent of these was carried out by Shelley *et al.*⁹⁶ In the case of neo-adjuvant hormone therapy, they found that when combined with RP, there was no improvement in overall survival. For example, Klotz *et al.* found 5-year overall survival levels were 88.4% for neo-adjuvant treatment patients and 93.9% for those who had received RP only ($p = 0.38$).⁹⁷ There was also no significant improvement in disease-free survival. When neo-adjuvant hormone therapy was combined with RP, there was, however, a significant

improvement in local pathological variables: positive surgical margin rate was significantly lower (relative risk [RR] 0.49, 95% CI 0.42 - 0.56, $p < 0.00001$), organ confinement was improved (RR 1.63, 95% CI 1.37 - 1.95, $p < 0.0001$), as was positive lymph node involvement (RR 0.66 95% CI 0.47 - 0.94, $p < 0.02$).

With regard to neo-adjuvant hormone therapy combined with RT, only one study has given data on patient overall survival.⁹⁸ The result for the whole patient population was not significant at 8 years but when sub-grouped, there was a significant difference in survival when those with a Gleason score of 2-6 were treated with neo-adjuvant therapy compared to those with a Gleason score of 7-10 (70% vs. 52%, $p = 0.015$). This significance was not maintained for 10-year overall survival (43% vs. 34% $p = 0.12$).⁹⁹ The group also looked at disease-specific survival, which was significantly improved in the neo-adjuvant group at 8 years (23% vs. 33%, $p = 0.05$) and remained so at 10 years (23% vs. 36%, $p = 0.01$).^{98, 99}

In the case of the combination of adjuvant hormone therapy and prostatectomy, results for overall survival vary between studies. Messing *et al.* reported that after a median of 7.1 years, overall survival was significantly better among patients who had received immediate anti-androgen therapy following RP compared to those who underwent RP alone ($p = 0.02$).¹⁰⁰ However, in a study by Wirth *et al.*, using flutamide as the adjuvant treatment for locally advanced lymph node positive prostate cancer, there was no difference in overall survival between the adjuvant therapy and control groups ($p = 0.92$).¹⁰¹

Results have also been varied for studies combining RT with adjuvant therapy for patients with localised or locally advanced carcinoma of the prostate. In a recent study, a follow-up of patients (median 8.2 years) found that risk of recurrence was significantly reduced for those treated with RT and androgen suppression therapy (AST).¹⁰² The risk was significantly decreased with each extra month of anti-androgen treatment when results were adjusted to take into account elements associated with increased risk of recurrence (increasing PSA level, high Gleason score (8,9 or 10), clinical stage T2 prostate cancer).

In the Early Prostate Cancer (EPC) trial programme, researchers are examining the impact of administration of bicalutamide 150 mg daily along with standard care (RP and/or RP or watchful waiting) as treatment for localised or locally advanced prostate cancer.¹⁰³ In the 7.4 year follow-up, bicalutamide 150 mg treatment was found to have no significant effect on progression-free survival for those patients with localised disease. However, when patients had locally advanced cancer, the treatment significantly enhanced progression-free survival when combined with all types of standard care (HR 0.69; 95% CI 0.58 – 0.82; $p < 0.001$). In the case of RT, additional bicalutamide 150 mg administration significantly increased overall survival in those with locally advanced disease (HR 0.65; 95% CI 0.44 – 0.95; $p = 0.03$).

In another study, RT was combined with adjuvant androgen suppression using the drug goserelin.¹⁰⁴ Absolute survival, at 10 years, was significantly higher for RT and goserelin than RT alone (49% vs. 39% respectively, $p = 0.002$). Similarly, Bolla *et al.* found that 5-year overall survival was significantly better when RT was combined with adjuvant hormone therapy (78% vs. 62%, $p = 0.001$).¹⁰⁵

1.6 Gene directed enzyme prodrug therapy

Gene directed enzyme prodrug therapy (GDEPT) or suicide gene therapy involves the administration of an enzyme-encoding transgene, the expression of which is controlled by a tumour specific promoter. A relatively low or non-toxic prodrug is introduced into the tissue, in this case the prostate, and is converted to its toxic form by the enzyme, leading to tumour specific cell kill.¹⁰⁶ Specific characteristics of prostate cancer make it a particularly suitable candidate for GDEPT. Although loss of function affects quality of life of the patient, the organ is not crucial for survival. Its location makes it relatively easy to access for direct intratumoral treatment strategies – specific drugs can be given through a transrectal, transperineal or transurethral route and biopsies can be taken to monitor progress. Prostate tumours express a highly specific protein, PSA, a marker for the disease that is extremely useful in assessing the success of the therapy. Existing phase I/II trials suggest that injections of the vector, as well as administration of the prodrug and follow-up monitoring, can all take place in an outpatient setting, reducing the potential cost of treatment.^{107, 108} There are nonetheless difficulties in treating

prostate cancer using gene therapy. Its multifocal growth pattern makes it challenging to target cancer cells and means that delivery of the vector to the whole gland is necessary. In addition, heterogeneity within the tumour cell micro-environment and intrinsic variations in genetic mutations causing the disease mean it can be hard to develop a therapy which will effectively kill all tumour cells.^{107, 108} The most commonly used GDEPT systems are discussed below.

1.6.1 Cytosine deaminase/5-fluorocytosine prodrug system

5-fluorouracil (5-FU) is currently used as a chemotherapeutic drug, in particular for the treatment of colorectal cancer. However, to be effective, high doses of the drug are required resulting in undesirable side-effects.¹⁰⁹ Therefore, a possible way to improve on this therapy is to introduce the drug using GDEPT. 5-fluorocytosine (5-FC) is a drug with low toxicity currently used to treat fungal infections. It has a half-life of 3 to 4 hours in patients with fully functioning kidneys.¹¹⁰ The cytosine deaminase enzyme from either yeast (yCD) or bacteria (bCD) converts 5-FC to the much more potent 5-FU.¹¹¹ 5-FU is further processed by endogenous enzymes to one of three pyrimidine antimetabolites: 3-fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP) is a thymidylate synthase inhibitor, preventing deoxythymidine triphosphate synthesis, a precursor of DNA; 5-fluorouridine-5'-triphosphate (5'-FUTP) disrupts RNA processing by incorporating into RNA in the place of UTP and 5-fluorodeoxyuridine-5'-triphosphate (5-FdUTP) incorporates into and damages DNA.^{109, 111-113}

The CD/5-FC system is capable of introducing a strong bystander effect, meaning surrounding cells will be affected, not just those that express the enzyme. 5-FC can freely diffuse between cells; it does not require direct cell-to-cell contact or gap junctional intercellular communication (GJIC).¹¹³⁻¹¹⁵ This is an important advantage: *in vitro* experiments involving transfected cells expressing CD, grown together with untransfected cells, showed that the number of transfected cells can be as low as 1-30% and still produce enough 5-FU to inhibit growth of the other untransfected cells.^{114, 116,}

117

1.6.2 *Herpes simplex virus thymidine kinase/ Ganciclovir prodrug system*

The GDEPT system, involving the enzyme herpes simplex virus thymidine kinase (HSVtk) and the prodrug 9-([2-hydroxy-1-(hydroxymethyl)methyl] guanine (ganciclovir or GCV), is currently the most extensively studied. GCV is phosphorylated to monophosphate GCV (GCV-MP) by the mammalian thymidine kinases but conversion is 1000 times faster with HSVtk.¹¹⁸ This is the rate-limiting step and mammalian guanylate kinase then further phosphorylates GCV-MP to GCV-DP (diphosphate) and GCV-TP (triphosphate).¹¹⁹ GCV-TP causes cell death through the inhibition of cellular DNA polymerase and incorporation into DNA in the place of dGTP during cell division, resulting in single strand breaks in the DNA.^{120, 121}

A drawback of the HSVtk/GCV system is that GCV-TP, being highly charged, is insoluble in lipid membranes. Therefore, the bystander effect will not occur through diffusion of GCV-TP into untransfected tumour cells. However, in preclinical studies transduction rates as low as 10% could still result in tumour regression.^{122, 123} This is thought to be due to a combination of transfer of the toxic drug across gap junctions between cells, as well as the release of GCV-TP in apoptotic vesicles from dying cells and their subsequent phagocytosis by transfected cells.^{123, 124} The immune system also plays a role in the bystander effect: administration of HSVtk/GCV treatment results in entry into the tumour of CD4+ and CD8+ T cells and macrophages and the heightened expression of the immune signalling molecules IL-2, IL-12, IFN- γ , TNF- α and GM-CSF.^{125, 126}

Attempts have been made to improve the bystander effect of HSVtk/GCV. For example, various groups have worked on ways to increase expression of connexin proteins, which have a role in development of gap junctions, through the use of pharmacological agents or the co-expression of the protein with HSVtk.¹¹³ Other groups have constructed fusions between HSVtk and proteins that stimulate intercellular import and export, such as 8-11 amino acids from the human HIV-1 TAT protein.¹²⁷ This fusion protein can move intercellularly, independent of gap junctions.

1.6.3 Other enzyme/prodrug systems

CD/5-FC and HSVtk/GCV are the two most commonly used GDEPT systems. However there are other systems which also have potential in the treatment of prostate cancer. For example, a Phase I/II clinical trial involved the treatment of localised prostate cancer with the GDEPT system nitroreductase/CB1954.¹²⁸ Nitroreductases convert CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] to hydroxylamide and further processing produces an alkylating agent which can form DNA cross-links independent of cell cycle.^{113, 128} This GDEPT system results in a strong bystander effect as activated CB1954 can move freely through cell membranes.¹²⁹ Toxicity was mild and 6 out of 18 patients had an increase in time to PSA progression.

Cytochrome P450 enzymes are found in the liver and can be used in GDEPT to achieve local, targeted cell death, as well as a large bystander effect.¹¹³ They convert the oxazaphosphorine prodrugs (ifosfamide and cyclophosphamide) to 4-hydroxy-forms which are unstable and decay to phosphoramidate mustard and acrolein. These are alkylating agents that result in DNA cross-links. Specific targeting of hypoxic cells has been investigated using the prodrug AQ4N which is metabolized to the cytotoxin AQ4 under hypoxic conditions.¹³⁰ GDEPT allows for the enhanced expression of cytochrome P450s in hypoxic cells to increase the level of AQ4N metabolism. Zhou *et al.* showed that if cytochrome P450-2C9 (CYP2C9) positive cells, H2C9, were co-cultured with the CYP2C9-negative prostate cancer cell line PPC-1 and treated with cyclophosphamide, the toxicity of the drug was four times higher than for PPC-1 cells alone, demonstrating a bystander effect.¹³¹

The use of bacterial purine nucleoside phosphorylase (PNPs) as part of a GDEPT system is also under investigation. The enzyme converts adenosine analogues to cytotoxic adenosine analogues leading to disruption of ATP-dependent reactions. Cell kill can therefore occur in both proliferating and non-proliferating cells.^{113, 132, 133} There is a very strong bystander effect as the drugs are membrane permeable.¹³² This GDEPT system has shown potential both *in vitro* and *in vivo*. For example, the PNP/6-methylpurine deoxyriboside system was demonstrated to be effective against PC3 xenografts in nude mice, resulting in tumour regression and increased survival.¹³⁴

Administration of the PNP gene to LNCaP-LN3 and PC3 xenografts in nude mice, alongside treatment with the prodrug fludarabine phosphate, resulted in a 100% increase in tumour doubling time and a greater than 50% inhibition of tumour growth.¹³⁵ In the TRAMP mouse model, in which the disease imitates human prostate cancer development, there was a significant decrease in tumour progression following PNP/fludarabine phosphate treatment.¹³⁶

1.7 GDEPT delivery vectors

Successful gene therapy treatment requires entry of the vector into the cells of interest. The most commonly used vector in gene therapy clinical trials is the adenovirus. As of June 2010, 23.8% of the 1644 trials undertaken involved this vector.¹³⁶ This overview of the field focuses on the adenovirus but there are multiple vectors, both viral and non-viral, that are being tested for use in GDEPT treatment. As well as the adenovirus, viral vectors include retrovirus, adeno-associated virus and herpes simplex virus. These vectors show relatively high levels of gene delivery but there are issues with patient safety and they can be hard to manufacture on a large scale.¹⁰⁶ Non-viral methods of gene delivery include using liposomes, nanoparticles, insertion of naked DNA and insertion of gold particle coated DNA using gene guns.^{106, 111, 137} Large scale production is easier for non-viral vectors. They are less likely to cause an immune response compared to viral vectors but they are also less efficient at gene transfer and result in only transient expression^{106, 107} The vector chosen depends on the nature of the disease being targeted. In the case of cancer, only short-term expression is required, compared to corrective gene therapy.¹³³ More specifically, the behaviour of the tumour is important. Prostate tumours are relatively slow growing so require a vector that can transduce both non-dividing and dividing cells.¹³³ The adenovirus meets both these criteria.

1.8 Optimal adenoviral delivery

Finding the most favourable route for adenoviral delivery is important to the success of GDEPT. This was examined in a canine model through the comparison of adenoviral transduction efficiency following its administration by three different routes: intravenous, intra-arterial and intraprostatic injections. The vector expressed β -

galactosidase and it was found that levels of this enzyme in the prostate were highest after intraprostatic injection.¹³⁸ De Weese *et al.* adapted techniques used for brachytherapy to administer the oncolytic adenovirus CV706 for the treatment of locally recurrent prostate cancer.¹³⁹ They used a computerised planning system to determine the best locations for stereotactic intraprostatic injection. A similar method was used by Barton *et al.* to determine the treatment design for two prostate cancer patients, based on their previous findings for gene expression distribution for an oncolytic adenovirus in naïve canine prostate and human tumour xenografts.¹⁴⁰

1.9 Combining GDEPT with radiation therapy

A combined treatment regime involving GDEPT as an adjuvant to radiation therapy has been suggested and could have numerous advantages. Gene therapy has the potential to improve the outcome of radiation therapy through a number of mechanisms. In a synergistic manner, radiation can also improve the efficacy of gene therapy and help overcome some of the hurdles to its progression to the clinic.

1.9.1 Radiosensitization

One potential benefit of combining the two treatment strategies is an increase in the radiosensitivity of the tumour. This would allow for a higher level of tumour cell death with lower doses, reducing toxic side effects. In the case of the CD/5-FC system there is evidence that 5-fluorouracil (5-FU) is a moderate radiosensitizer.¹⁴¹ Although the mechanisms that control this are not fully understood, it is thought to involve FdUMP, which inhibits thymidylate synthase, thereby preventing DNA repair following irradiation.^{141, 142} Also, gene therapy is effective during the S phase of the cell cycle when DNA synthesis is taking place. This is the stage when cells are normally more resistant to radiation therapy.⁵⁹

The effect of 5-FU on radiosensitivity appears to vary between different tumour types, although it has been shown to have an effect in prostate tumour cells. Smalley *et al.* compared various mammalian cell lines for the inducement of radiosensitivity by 5-FU; a continuous exposure to radiation during 5-FU treatment resulted in radiosensitization in DU145 prostate cancer cells as well as V-79 (Chinese hamster lung fibroblast) and

WiDr and HT-29 (human colon carcinoma) cell lines but not in HeLa cells.¹⁴³ Pulse exposure (radiation given halfway through 5-FU treatment) resulted in radiosensitivity in just the DU145 cells.

GCV is phosphorylated by HSVtk and acts as a radiosensitizer as it is a nucleotide analogue that prevents DNA replication, therefore stopping the repair of DNA damage caused by radiation.¹⁴⁴ The combination of HSVtk and GCV with radiation has been shown to enhance treatment efficacy for prostate cancer *in vivo*.¹⁴⁴ There was a significant rise in the inhibition of tumour growth and increase in survival times in a RM-1 prostate cancer mouse model when the therapies were united, compared to radiation or gene therapy alone. Subsequently, mice were given a tail vein injection of RM-1 to initiate lung metastases formation, as well as the original subcutaneous injection. It was found that radiation alone had no effect on lung nodule number whereas the combined therapy resulted in a reduction of 87% (HSVtk and GCV alone reduced nodule number by 37%).

Although further work is necessary to better understand the processes that control development of radiosensitivity following drug treatment, it has the potential to enhance the cytotoxic effects of radiotherapy and improve its efficacy while also possibly allowing for a reduction in tumour dose.

1.9.2 Improved viral uptake

It has been demonstrated that radiation treatment increases the level of uptake of adenovirus into mammalian cells as well as tumour xenografts. Stevens *et al.* showed that irradiation of mammalian cells with up to 7 Gy improved subsequent adenoviral transduction by up to 40-fold in the human neoplastic cell line A549.¹⁴⁵ Notably, the effect was dose dependent and expression of the transgene, *lacZ*, lasted longer in irradiated cells and this was shown to be secondary to the integration of the adenovirus into the host DNA. This phenomenon was also observed by Zhang *et al.* following exposure of mammalian cell lines and rat hepatocytes to 4 Gy of radiation prior to transduction with a green fluorescent protein (GFP) expressing adenoviral vector – there was an associated 27.1-fold increase in GFP expression.¹⁴⁶ *In vivo*, the level of

transgene expression (this time *lacZ*) in rat intrahepatic colon carcinoma xenografts was also raised by combining it with radiation (25 Gy).

The same group went on to show the importance of Dynamin 2, a membrane-associated protein, in this process. In cells treated with an antibody to Dynamin 2, the level of viral uptake and gene transfer in response to radiation was significantly reduced in rat hepatocytes and human colon carcinoma cells.¹⁴⁷ In contrast, antibodies for coxsackie B virus and adenovirus receptor (CAR) or integrin α_v had little or no effect. mRNA and protein levels of Dynamin 2 were shown to rise after irradiation. The introduction of siRNA specific to Dynamin 2 led to a significant drop in the amount of viral uptake and gene transfer in radiation treated cells. In colon cancer xenografts, Dynamin 2 levels were higher following irradiation, as was the amount of viral uptake. The results of this study point to an important role for Dynamin 2 in the radiation-induced rise in adenoviral uptake. Egami *et al.* demonstrated that in pancreatic tumour cells, as well as subcutaneous tumours in nude mice, transgene expression levels following infection with an adenoviral vector were higher when radiation was also administered.¹⁴⁸ These results show that combining adenovirus administration with radiation therapy has the potential to improve transgene expression levels, thereby increasing the efficacy of GDEPT.

Clinical trials of GDEPT in combination with radiation therapy

1.9.3 GDEPT for the management of local failure following radiation therapy

GDEPT was initially investigated for its potential in the treatment of locally recurrent prostate cancer to increase PSA doubling time and potentially delay the time to initiation of androgen ablation. Table 1.1 summarises the results of the clinical trials involving GDEPT for the treatment of prostate cancer. The first phase I clinical trial to demonstrate the potential benefit of GDEPT in locally recurrent prostate cancer was carried out in Baylor College of Medicine, Houston in 1999.¹⁰⁸ Selected patients had local recurrence of prostate cancer, as demonstrated by biopsy, at least one year after definitive irradiation therapy, along with a rising PSA level but no evidence of metastasis of the tumour. Herman *et al.* used a replication-deficient adenovirus to

transfect patients with HSVtk. A series of escalating concentrations of the adenovirus were administered to patients intraprostatically followed by intravenous injection of GCV two weeks later. Administration of the therapy resulted in a decline of more than 50% in the serum PSA level of 3 of the 18 patients. For the patient at the highest dose level this lasted for more than a year. Moreover, the safety of this treatment was established, with the majority of patients displaying minimal toxicity or none at all, although one patient who received the highest dose of the adenovirus vector (1×10^{11} infectious units) experienced grade 4 thrombocytopenia and grade 3 hepatotoxicity.

This study led to an extended phase I/II trial using the ADV/HSKtk adenovirus to treat 36 patients with clinically localised recurrent prostate cancer at least 18 months after radiotherapy and increasing serum PSA levels, including the 18 patients from the previous trial.¹⁴⁹ 27 of the patients showed a drop in serum PSA after vector infection and administration of GCV, with a mean reduction of 28%. The average time taken for PSA levels to return to their original value was calculated at 8 months. Treatment effect on PSA doubling time (PSADT) was examined in 22 of the patients (those who had not been excluded due to insufficient data or the development of metastatic disease), with a reported increase in PSADT from 15.9 months to 42.5 after gene therapy ($p = 0.0271$). PSADT has been shown to have strong prognostic power and is predictive of metastasis formation and prostate cancer-specific mortality.¹⁵⁰⁻¹⁵³

In separate studies, oncolytic replication-competent adenoviruses have also been investigated for the treatment of localised prostate cancer. The first clinical trial involving a prostate-specific oncolytic adenovirus was set up by investigators at John Hopkins Medical Institute in 2001.¹³⁹ The adenovirus was CV706, originally reported by Rodriguez *et al.*, a replication-competent, E3-deleted Ad5 adenovirus that contains a promoter-enhancer construct from the human PSA gene, thereby restricting replication to PSA-expressing cells.¹⁵⁴ All patients chosen had locally recurrent prostate cancer following EBRT, as well as increasing PSA levels. Treatment was found to be safe with low toxicity – most men experienced only mild adverse events (AEs). Grade 3 toxicities did occur in five men but these were all reversible and were not deemed to be directly related to the drug itself. In 65% of patients, PSA dropped by $\geq 30\%$ compared

to levels pre-treatment. Notably, 25% of patients showed an even greater reduction ($\geq 50\%$) in PSA, all of whom had received the highest or second highest dose of CV706, suggesting the response was dose dependent. Evidence for *in vivo* adenovirus replication was also seen as there was a secondary peak in levels of CV706 DNA in the circulation of most patients approximately 3 days after treatment.

A logical progression for this cancer gene therapy approach was to combine the oncolytic adenovirus with GDEPT. Conceptually, the use of replication-competent adenovirus would result in higher levels of gene expression and increase the spread of the virus into nearby tumour cells.⁹⁴ This was first tested in a Phase I study by Freytag *et al.* in which the adenovirus (Ad5-CD/TKrep) was used to deliver a CD/HSVtk fusion gene.¹⁵⁵ The expression of these genes was expected to enable the activation of non-toxic prodrugs to their toxic forms. Furthermore, both suicide gene systems (CD/5-FC and HSVtk/GCV) increase sensitivity to radiation in addition to the oncolytic properties of the adenovirus. The strategy was tested in patients who had evidence of recurrent prostate cancer at least one year after definitive radiation therapy, as well as rising serum PSA levels (but < 20 ng/ml) and an absence of metastatic disease. Sixteen patients, in four groups, were given escalating doses of the virus up to 10^{12} virus particles. Two days subsequently, patients began 1-2 weeks of 5-FC and GCV therapy. Toxicity was low, with most of the AEs classed as grade 1 (82%) or grade 2 (12%). Only one AE, a grade 1 thrombocytopenia, necessitated a change to the treatment plan. Of the 6% of AEs that were grade 3, all were self-limiting. Of the sixteen patients in the trial, 7 (44%) showed a drop in serum PSA levels of $\geq 25\%$ and 3 (19%) showed a $\geq 50\%$ decrease for at least 4 weeks. The longest duration of PSA reduction was 4 months. In most cases the reduction in PSA levels was greatest during 5-FC/GCV therapy, followed by a levelling off of PSA levels or a much slower decrease until the virus was no longer present in the prostate, indicated by an absence of Ad5-CD/TKrep DNA in the blood. Therefore, Freytag *et al.* concluded that the majority of antitumour activity was a result of the suicide gene systems and that the subsequent, less dramatic, decrease in PSA represents the oncolytic activity of the adenovirus. The success of the therapy was also assessed using prostate needle biopsies after 2 weeks where

coagulative necrosis, indicating tumour destruction, was seen in several cases, with two patients remaining negative for adenocarcinoma one year after therapy.

Recently, a five year follow up has been carried out by Freytag *et al.* on their trial of the replication-complement adenovirus, Ad5-CD/TK rep .^{155, 156} As mentioned previously, the decrease in serum PSA levels was brief (< 6 months). However the results from the follow-up study are promising. No long term side-effects were observed. PSADT was used as a measure of the effectiveness of the therapy. Of the sixteen original participants, fourteen were evaluable. It was found that PSADT improved from a mean of 17 to 31 months (median 16 to 22 months) ($p=0.014$) following treatment. It is unclear why the PSADT continues to increase, even in the absence of the adenovirus; Freytag *et al.* suggest anti-tumour immunity may have been contributory. In support of this, previous studies with mice models have shown that CD and HSVtk suicide gene therapy results in T cell-dependent antitumour immunity.^{126, 157} Given the possible activation of an immune response, Freytag *et al.* believe it suggests this therapy may have the potential to be used to treat metastatic as well as local disease. The long term therapeutic effect of the treatment was also assessed by calculating the extent of the delay in the initiation of salvage androgen suppression therapy and was an average of 2.1 years.

In summary, the results of the clinical trials examining the benefit of GDEPT in localised recurrent prostate cancer are encouraging. The safety profile has been established and the benefit to patients has also been demonstrated. GDEPT could therefore be an option in follow-up treatment if the initial therapy fails. An alternative therapy that could delay or even replace androgen suppression therapy for patients with rising PSA levels following definitive therapy could greatly improve quality of life.

1.9.4 GDEPT to improve radiosensitivity in newly diagnosed localised prostate cancer

The success of treatment for localised prostate cancer is highly variable. Although rates of long term disease-free survival are high for low-risk prostate cancer following radiation therapy (~80%), they are much lower for those who have intermediate or

high-risk forms of the disease (25-75%).¹⁵⁸ Therefore, ways to improve the initial therapy for localised prostate cancer and prevent metastatic tumour formation are necessary. The potential of GDEPT has been examined in the case of newly diagnosed tumours.

The first clinical trial to investigate the potential of combining radiation therapy with GDEPT was performed by Teh *et al.* in 2001.¹⁵⁹ The phase I/II study involved 30 patients divided between three arms: Arm A consisted of 13 low-risk patients (PSA < 10ng/ml, Gleason score < 7, Stage T1-T2a); the 14 patients in Arm B were high-risk (PSA ≥ 10ng/ml, Gleason score ≥ 7, Stage T2b-T3) and the 3 Arm C patients had Stage D1 cancer and therefore had positive pelvic lymph nodes. The patients were treated with a combination of ADV/HSVtk by intraprostatic injection and the prodrug valacyclovir given 24 hours later and for 14 days, together with a mean dose of 76 Gy of radiation given by intensity-modulated radiation therapy (IMRT). In addition, Arm C patients received 45 Gy to the pelvic lymphatics and those in Arms B and C were treated with androgen deprivation therapy. The initial aim was to assess the safety of the treatment regime. Toxicity levels were found to be low and there was no increase in the amount of genitourinary or gastrointestinal injury compared to IMRT alone. Most AEs were grade 1 or 2. One patient experienced a grade 3 rise in the liver enzyme ALT but this returned to normal without intervention.

The results of an extended trial and follow-up of patients were later published by Teh *et al.*¹⁶⁰ In the study PSA and biopsy results for 59 patients divided between Arm A (29 patients), Arm B (26 patients) and Arm C (4 patients) were reported. PSA measurements showed biochemical control had been achieved in all patients in Arms A and B (median follow-up 13.4 and 13.9 months respectively). Three of the four patients in Arm C, however, experienced biochemical failure. The disease had metastasised to the bones in two of these men and to the paraaortic lymph nodes in the other. For arm A and B, the biopsy results showed an increase in the carcinoma-negative samples over 24 months. The group recognised there was a chance that the biopsies failed to detect tumour and that more long-term follow-up was needed but the initial results of the trial were positive.

Freytag *et al.* further investigated the Ad5-CD/TKrep gene therapy treatment and examined its effects in combination with 3D-CRT in patients with newly diagnosed intermediate to high-risk prostate cancer at stage T1c to T3.¹⁵⁸ The mean Gleason score was 8, with a range of 6–9. The mean serum PSA was 12.9 ng/ml, with a range of 2.5–30.9 ng/ml. Patients had no evidence of metastatic disease, as determined by bone scan and CT scan of the abdomen and pelvis. In this study, Ad5-CD/TKrep was administered to 15 patients by intraprostatic injection. This was followed two days later, with the administration of 5-FC and valganciclovir (vGCV, an orally administered alternative to ganciclovir), as well as 70-74 Gy of radiation therapy for 1, 2 or 3 weeks. In keeping with the previous trial, AEs were predominantly mild (65% grade 1) or moderate (29% grade 2). The common side effects of 3D-CRT, bladder and bowel toxicities, were no more frequent in this trial, despite the inclusion of an oncolytic adenovirus and gene therapy that increases radiosensitivity. Transgene expression in the prostate lasted up to 3 weeks after injection. The mean PSA half-life for those receiving prodrug therapy for greater than 1 week was shorter than for 3D-CRT alone (0.6 vs 2.4 months).

Freytag *et al.* continued their work by developing an improved, second-generation adenovirus and testing it in combination with IMRT in men with newly diagnosed prostate cancer.¹⁶¹ The new adenovirus, Ad5-yCD/mutTKSR39rep-ADP, contains the yeast, rather than the bacterial CD gene and the mutant gene SR39 HSVtk, both of which produce more catalytically active enzymes. Experimental data suggested an increase in radiosensitivity and chemotherapeutic effect using this adenovirus. In addition, the vector codes for the adenovirus death protein (ADP) which improves cell killing and spread of the virus. In a phase I trial, 9 patients received 10^{11} , 10^{12} or two doses of 10^{12} virus particles of the second generation adenovirus. Three days after each injection, they began a 13 day course of 5-FC, vGCV and 74Gy IMRT. There was no increase in toxicity compared to the original first generation adenovirus, in spite of an increase in catalytic activity. To assess the merit of the combination of suicide gene therapy with radiotherapy, the result from the trials of Ad5-CD/TKrep and Ad5-yCD/mutTKSR39rep-ADP were merged and compared to the expected results for

radiotherapy alone. In $\geq 40\%$ of the patients, one would expect a positive prostate biopsy following radiation therapy. However, in the case of these trials, in which radiotherapy was used alongside suicide gene therapy, only 22% of the evaluable patients had a positive final biopsy ($p=0.038$). The results were more informative when the patients were divided by prognostic risk category: none of the 12 intermediate risk patients were positive for adenocarcinoma at biopsy, which is significantly different from the expected result of $\geq 30\%$ ($p<0.01$). On the other hand, the result for the high risk group (45%) was not significantly different from the expected level using radiotherapy alone (56%) ($p=0.72$).

An adaptive seamless randomised Phase II/III trial to follow-up these results has been approved and the group are presently recruiting patients.¹⁶² 280 patients will be divided into two arms. Arm one will receive one dose of 10^{12} virus particles of Ad5-yCD/*mutTK_{SR39}rep*-ADP intraprostatically, followed by a two week course of 5-FC, vGCV alongside an eight week course of 80Gy IMRT. Those in Arm two will receive IMRT alone. The Phase II component of the trial will involve assessment of toxicity in the first 21 patients in Arm 1, 90 days into the trial. Efficacy will be measured using time free from biochemical and/or clinical failure as well as prostate biopsies at year 2 to assess local tumour control and analysis of overall survival rates. It will be the first randomized controlled trial using gene therapy to treat non-metastatic prostate cancer.

The results of previous trials suggest that GDEPT can be safely used alongside radiation therapy and when combined, it has the potential to improve treatment outcome compared to radiation therapy alone. It is encouraging that this treatment is now moving into a Phase II/III trial.

1.9.5 GDEPT for the treatment of metastatic prostate cancer

A limited number of studies have investigated the potential of GDEPT to improve treatment of metastatic disease. Kubo *et al.* carried out a phase I clinical trial using osteocalcin promoter-driven HSVtk (Ad-OC-hsv-TK).¹⁶³ The trial involved 11 patients – two with local recurrence of cancer after surgery, four with lymph node metastases and five with osseous metastases. The men had previously been treated with surgery,

radiation therapy, chemotherapy and/or hormonal therapy. Each patient received two intralesional injections of Ad-OC-hsv-*TK* at one of three dose levels (5×10^9 , 5×10^{10} and 5×10^{11} particles/injection) and 1g of valacyclovir taken orally every day for 21 days. The treatment was shown to be relatively safe with most of the AEs at grade 1 or 2. Using TUNEL staining, an increase in cell death was seen in the treated lesions of seven of the patients (63.6%). In one patient, the treated lesion was stabilised for 317 days, whilst another patient experienced a decrease of $> 50\%$ in PSA level. The remaining patients did not show a significant fall in PSA or reduction in tumour size.

This study led to a phase I/II clinical trial of Ad-OC-hsv-*TK* / VAL gene therapy.¹⁶⁴ 6 patients were involved, all of whom had bone metastases and relatively high PSA levels (46.6 – 455.5 ng/ml). The patients had been treated with hormone therapy and chemotherapy already, as well as radical prostatectomy and/or radiation therapy in some cases. Once again the safety of the treatment was demonstrated. One of the 6 patients had a distinct decline in PSA (318.3 to 4.9 ng/ml) and time to PSA progression was one year. CT scans of this patient showed a decrease in the metastatic density of the lesion at day 180. Following PSA failure after gene therapy treatment docetaxel and estramustine combination chemotherapy was administered to three of the patients. This was effective in all three, with time to PSA progression at 21, 7 and 4 months.

In this limited range of trials, the relative safety of GDEPT in patients with metastatic prostate cancer was demonstrated. In addition, there have been some promising results in terms of the benefit of the treatment to the patients. Currently, available therapies are merely palliative so any improvement in this is desirable.

It is clear that an important consideration in the development of a gene therapy strategy is the decision on what patient cohort should be targeted. The gene therapy clinical trials involving both localised and metastatic disease have been discussed. However improvements in systemic treatments for metastatic cancer are only necessary because primary treatment fails in a high proportion of cases.⁹⁴ Therefore, it is logical to seek improvements in treatment of localised cancer and restrict its spread. The clinical trials discussed here suggest that treatment of localised disease with radiotherapy could be

more effective if combined with GDEPT. The future of GDEPT is likely to be as part of a multi-modal treatment strategy in which it works to improve the efficacy of the current methods of prostate cancer treatment.

	Vector/Enzyme + Prodrug(s)	Phase	N	Toxicity	Results	References
Newly diagnosed localised prostate cancer	ADV/HSV- <i>tk</i> + vGCV	I/II	30	Most AEs grade 1 or 2. Grade 3 ALT elevation in 1 patient. Grade 3 acute genitourinary toxicity in 1 patient.	The clinical outcome of the treatment was assessed at a later date. ¹⁶⁰	¹⁵⁹
	ADV/HSV- <i>tk</i> + vGCV	I/II	59		Biochemical control in all low and intermediate-risk patients, as well as negative biopsies. Biochemical failure and metastasises in 3 (75%) of the high-risk patients.	¹⁶⁰
	Ad5-CD/TK <i>rep</i> + 5-FC + vGCV	I	15	Most AEs grade 1 (65%) or grade 2 (29%).	Mean PSA half-life shorter with prodrug therapy (> 1 week) than for 3D-CRT alone (0.6 vs 2.4 months).	¹⁵⁸
	Ad5-yCD/ <i>mut</i> TKSR39 <i>rep</i> -ADP + 5-FC + vGCV	I	9	Most AEs grade 1 or 2 (92%)	(Results combined with earlier trial ¹⁵⁸) 0 of 12 intermediate-risk patients had positive biopsies. Significantly different from the expected result of $\geq 30\%$ for radiation therapy alone.	¹⁶¹
Locally recurrent prostate cancer	ADV/HSV- <i>tk</i> + GCV	I	18	Mild toxicity in 4 patients (grade 1-2). Significant toxicity in 1 patient at highest dose.	Drop in serum PSA levels of 50% or more in 3 patients. Lasted up to one year.	¹⁰⁸
	ADV/HSV- <i>tk</i> + GCV	I/II	36	Mild toxicity in 13 patients (grade 1-2). Significant toxicity in 2 patients at highest dose (grade 3-4).	Drop in serum PSA levels in 27 patients (Mean 28%). PSADT increased from 14.3 months to 42.5.	¹⁴⁹
	Ad5-CD/TK <i>rep</i> + 5-FC + GCV	I	16	Most AEs grade 1 (82%) or grade 2 (12%)	Drop in serum PSA levels of $\geq 25\%$ in 7 patients (44%) and $\geq 50\%$ in 3 patients (19%).	¹⁵⁵
					5-year follow-up: PSADT improved from a mean of 17 to 31 months. Delay in the initiation of androgen suppression therapy (average 2.1 years).	¹⁵⁶
Metastatic prostate cancer	Ad-OC-hsv-TK + vACV	I	11	Most AEs grade 1 or 2. Grade 3 lymphopenia in 2 patients. Grade 3 neutropenia in 1 patient.	Increase in cell death in the treated lesions of 7 patients. 1 patient had a decrease of > 50% in the level of PSA.	¹⁶³
	Ad-OC-hsv-TK + VAL	I/II	6	Most AEs grade 1 or 2. Grade 3 lymphopenia in 3 patients.	Decline in PSA (318.3 to 4.9 ng/ml) in 1 patient with time to PSA progression of one year. (Chemotherapy given to 3 patients following failure of GDEPT and was effective in all 3).	¹⁶⁴

Table 1.1: GDEPT trials for the treatment of prostate cancer. Summary of clinical trials that used GDEPT to treat localised, locally recurrent and metastatic prostate cancer, including the toxicity and the outcome of the therapy.

3D-CRT - 3D-conformal radiation; 5-FC - 5-fluorocytosine; Ad5 - adenovirus type 5; AEs - adverse events; ADP - adenovirus death protein; ADV - adenovirus; CD - cytosine deaminase; GCV - ganciclovir; HSV-*tk* - herpes simplex virus thymidine kinase; OC - osteocalcin; PSA - prostate specific antigen; PSADT - prostate specific antigen doubling time; *rep* - replication competent; vACV - valacyclovir; VAL vGCV - valganciclovir; yCD - yeast cytosine deaminase

1.10 Transcriptional targeting of GDEPT

Most of the clinical trials for GDEPT treatment of prostate cancer have involved the use of non-specific promoters to achieve high levels of enzyme gene expression. However this approach may lead to off-target toxicity: following intraprostatic injection of adenoviral vectors in a canine model, the sequences could be detected in other organs, mainly the vas deferens and bladder.¹⁶⁵ Tissue/tumour-specific activation of the prodrug can be achieved through the use of promoters that restrict expression of the enzyme to target cells. This approach should mean a reduction in side effects as it lowers toxicity to healthy tissues.^{133, 166} A large range of different tissue, tumour and exogenously controlled promoter types have been examined including those that relate to cell cycle and glucose response, or those that can be controlled by heat or certain drugs.¹⁶⁷ The most relevant for this project are prostate, hypoxia and radiation-specific promoters.

1.10.1 Prostate-specific

Different organ-specific promoters have been tested for the development of GDEPT systems to treat prostate cancer. The tissue-specific PSA gene promoter has been utilized as PSA is produced exclusively by the epithelial cells of the prostate¹⁶⁸ and it can be coupled with the PSA enhancer to further improve specificity. Latham *et al.* found a tandem duplication of the PSA enhancer upstream of the promoter increased expression by 50 times while still being prostate-specific.¹⁶⁹ They also demonstrated that the level of downstream gene expression was increased with addition of androgen and levels approached that of the constitutively active CMV-driven promoter in LNCaP but not a bladder cell line. Further attempts to improve the efficiency of the PSA promoter/enhancer have included its combination with the Cre-loxP DNA recombination system which enhanced activity of the promoter.¹⁷⁰ It was used to control CD expression and showed reduced LNCaP tumour growth *in vivo*.

The prostate-specificity of the prostate stem cell antigen (PSCA) promoter to control a lentiviral vector has been tested recently.¹⁷¹ Luciferase expression in a range of cell lines was restricted to the prostate cancer lines and was increased with androgen stimulation. When a vector expressing HSVtk under the control of PSCA

was used, GCV related toxicity showed efficacy and prostate-specificity *in vitro* and *in vivo*.

Prostate specific membrane antigen (PSMA) is a membrane protein expressed specifically in prostate cells and metastases. PSMA promoter/enhancer controlled expression of luciferase was tested in a number of cell lines but was detected only in the prostate cancer lines LNCaP and C4-2.¹⁷² When used to control CD expression, 5-FC sensitivity was seen in C4-2 *in vitro* and in C4-2 tumours in mice, resulting in elimination of the tumour. A chimeric enhancer produced using regulatory elements of PSA and PSMA also showed high prostate-specificity.¹⁷³ A double suicide gene therapy strategy was tested in LNCaP cells, in which the PSMA promoter/enhancer controlled uracil phosphoribosyltransferase (UPRT) in one vector and CD expression in another vector.¹⁷⁴ UPRT is part of the pathway that converts 5-FU to 5-FdUMP, which inhibits thymidylate synthase to prevent DNA synthesis. This strategy was shown to be more effective than treatment with the CD expressing vector alone.

Probasin has also been tested as a prostate-specific promoter for the control of a GDEPT system. Probasin-controlled and CMV-controlled GFP-expressing lentiviruses were compared and the prostate-specificity of probasin was demonstrated both *in vitro* and *in vivo*.¹⁷⁵ PSA and PB were fused to the *E. Coli lacZ* gene and tested for tissue specificity in a canine model.¹⁷⁶ Despite some dissemination of the vectors, *lacZ* expression was detected solely in the prostate for both promoters, in contrast to a control vector with non-specific promoter activity, where *lacZ* was detected in all tissues tested.

For the treatment of metastatic disease, when the tumour is no longer organ-confined, researchers have attempted to target treatment with GDEPT using the promoter of osteocalcin, a noncollagenous bone protein. Osteocalcin expression is upregulated in bone metastatic prostate cancer and the osteoblast cells at the site.^{177, 178} It was hypothesised that this property would enable the vector to target the tumour-associated stroma cells as well as the tumour itself. A phase I/II clinical trial has been carried out demonstrating safety and potential patient benefit¹⁶⁴ (section 1.9.5).

1.10.2 Hypoxia-responsive

Hypoxia is a significant factor in determining cancer prognosis, both because of its role in the development of a more aggressive phenotype and resistance to radiotherapy and chemotherapy. Research has been carried out to develop hypoxia-targeted therapy through the use of the unique characteristics of hypoxic cells.^{49, 179} In conjunction with standard therapies, this has the potential to greatly improve prognosis and overcome the current treatment problems associated with hypoxia. One example of this is gene therapy in which expression of the therapeutic gene is controlled by a tumour-specific trait, in this instance, the high expression of HIF-1 in hypoxic tumour cells but not in normal cells.⁴⁵ The use of hypoxia response elements from various genes have been tested as a means of achieving hypoxia-specific expression, including vascular endothelial growth factor (VEGF), erythropoietin (Epo), and phosphoglycerate kinase-1 (PGK1).¹⁶⁷

Use of a hypoxia-responsive promoter containing 5 HREs to control CD expression has been shown to be effective, inducing substantial cytotoxicity and enhancing the efficacy of radiation therapy in tumour xenografts of HeLa cells.¹⁸⁰ GAPDH HREs were used for the first time in a GDEPT system when 8 copies were used to control CD expression.¹⁸¹ This was tested alongside another vector containing 5 copies of the VEGF HRE. Both were effective in the prostate cancer cell lines DU145 and 22Rv1 on their own or in combination with clinically relevant doses of radiation. Harvey *et al.* showed the potential of a nitroreductase (NTR)/CB1954 prodrug-activating system under the control of 5 repeats of the VEGF HRE linked to the minimal cytomegalovirus promoter.¹⁸² It was tested *in vitro* and *in vivo* in human HCT116 xenografts in nude mice, where there was a significant decrease in tumour growth. A recent study found that a hypoxia-induced cytosine deaminase/5-fluorocytosine system delayed tumour growth and increased survival rates in mice being used as a lung tumour model.¹⁸³ This was tracked using the hypoxia imaging biomarker Tc-99m-HL91.

1.10.3 Radiation-inducible

As mentioned in section 1.9.1, the combination of GDEPT with radiation therapy has the potential to improve both treatment strategies. Both 5-FU and phosphorylated GCV, commonly used drugs in GDEPT, act as radiosensitizers

when combined with radiation treatment. Research has investigated the regulation of enzyme gene expression using a radiation responsive promoter as a method of transcriptional targeting. Numerous genes are known to be radiation-responsive, such as early growth factor-1 (*Egr-1*), *NF-κB* and cyclin dependent kinase inhibitor (*p21^{WAF1/CIP1}*).¹⁶⁷ However, in many cases, the mechanisms controlling this expression have yet to be fully elucidated.

Study of the *Egr-1* promoter identified multiple copies of a 10-bp sequence, CC(A/T)₆GG, termed the CArG element, responsible for the radiation-inducibility of the promoter.¹⁸⁴ Using the promoter of *Egr-1* for the radiation-induced control of GDEPT has shown success but the promoter has also been found to be “leaky”, resulting in expression even without irradiation.¹⁸⁵ Anton *et al.* used the bovine growth hormone polyadenylation signal as insulating sequences in the adenoviral vectors to reduce background expression in the absence of radiation treatment.¹⁸⁵

The promoter of the tissue plasminogen activator gene (t-PA), which expresses a serine protease involved in converting plasminogen to plasmin and has been shown to be activated by radiation, was inserted upstream of GFP.¹⁸⁶ The potential of t-PA as a radiation-inducible promoter was demonstrated in several prostate cell lines as well as HMEC-1 and *ex vivo* rat tail artery in response to a range of radiation doses (2, 4, 6 Gy). GFP expression was induced in all experimental models used. The *p21^{WAF1/CIP1}* promoter was shown to be tumour specific, showing high levels of expression in tumour cells line but relatively little in normal cell lines.¹⁸⁷ Additionally, it could be further induced by external beam radiation, radionuclides and hypoxia.

Morii *et al.* constructed a radiation-responsive promoter suitable for prostate cancer cells.¹⁸⁸ They used the *cis*-acting elements of five genes known to be regulated by radiation in prostate cancer cells: *NF-κB*, AP-1, Oct-1, p53 and Nrf-2. These were linked to the TATA-box sequence from the human heme oxygenase-1 gene promoter. From the promoter library, they selected the clone with the highest levels of enhancement following irradiation and subsequently used the introduction of random mutation to improve its activity. Luciferase expression, under the control of the promoter, was enhanced in LNCaP cells following radiation treatment. The promoter also enhanced 5-FC cell kill when upstream of a fusion gene expressing

cytosine deaminase and uracil phosphoribosyl-transferase. The group later went on to show that this promoter can also be activated by the drug doxorubicin.¹⁸⁹ This research indicates artificial construction of promoters may allow more specific and effective transcriptional targeting of GDEPT.

1.11 Hypothesis & project aims

If prostate cancer is detected early, while still localised, current treatment strategies are successful in a high percentage of cases. Cancer-specific survival rates are 87% and 92% for radiation therapy and radical prostatectomy respectively.⁷¹ However treatment failure is still a problem and curative options for treating local recurrence are limited. Therefore the search for new treatment options that could be given alone or as an adjuvant to current therapies is a worthwhile endeavour. GDEPT has potential as a treatment for prostate cancer and as an adjuvant to radiation therapy. This project involves the investigation of the potential of one GDEPT system, the cytosine deaminase (CD)/5-fluorocytosine (5-FC) system, through use of a vector, pPPE-CD, which contains the PSA promoter and enhancer upstream of the CD gene. Previous work in Prostate Molecular Oncology Group has demonstrated that pPPE-CD-driven CD expression is upregulated by treatment with the androgen DHT. It is hypothesised that cross-talk between hypoxia and androgen demonstrated in LNCaP¹⁹⁰ may also affect expression levels of CD with pPPE-CD. This is tested in two cell line models of prostate cancer: primary tumour (22Rv1) and metastatic disease (LNCaP). Investigation of hypoxia is important as it has been implicated in resistance to treatment and targeting cells that are resistant to current treatments may improve outcome. In this project, pPPE-CD activity is also tested in combination with radiation treatment to investigate the potential of this GDEPT system as an adjuvant. This has permitted the assessment of the efficacy of a combined treatment strategy when compared to GDEPT or radiation treatment alone.

As well as an investigation of the pPPE-CD/5-FC GDEPT system, this thesis also explores the models currently available for the study of prostate cancer. As a model for primary prostate cancer, the 22Rv1 cell line lacks the high PSA expression levels seen *in vivo*. Therefore, the cell line has been stably transfected with the androgen receptor to try to increase PSA expression levels. In another attempt to

improve on the currently available techniques, 22Rv1 spheroids have been developed and characterised. 3D tumour models may better reflect the tumour microenvironment *in vivo* and therefore be a better system in which to test potential drugs or therapies compared to the traditional monolayer culture of cells. The tumour spheroids are used to explore further the potential of the GDEPT system under various conditions.

Aims

To find a suitable endogenous control gene for qRT-PCR experiments involving hypoxic and aerobic samples.

To further characterise the optimal conditions for pPPE-CD-mediated cell kill and to investigate the influence of crosstalk between hypoxia and androgen on the activity of the vector.

To determine the effects of a combination of the pPPE-CD/5-FC system with radiation treatment.

To modify the current 22Rv1 cell line model of prostate cancer to reflect better the *in vivo* level of androgen receptor expression and use it as a model with which to assess the activity of the vector.

To generate a spheroid-based model of prostate cancer for testing of the pPPE-CD vector in a system that more accurately mimics *in vivo* conditions.

Chapter 2

Materials & Methods

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

Six prostate cancer cell lines were used during the course of this project: 22Rv1, LNCaP, DU145, PC3, PWR-1E and RWPE-1. 22Rv1 and LNCaP express AR mRNA and protein. They were originally obtained from the American Type Culture Collection (Teddington, UK). LNCaP, DU145 and PC3 are derived from lymph node, brain and bone metastases of prostate carcinoma respectively. 22Rv1 is a primary tumour cell line, derived from a prostate carcinoma xenograft. PWR-1E and RWPE-1 are normal prostate epithelial cells.

2.1.2 Growth conditions

Cells were grown in RPMI 1640 GlutaMax (Gibco, USA), supplemented with penicillin (50 U/ml), streptomycin (50 U/ml) and 10% foetal bovine serum (FBS, Gibco). Cells were grown in 75 cm² (T75) flasks (Nunc, Denmark) in a humidified incubator (Forma Steri-Cycle CO₂ Incubator, Thermo Scientific USA) at 37°C with 5% CO₂. For LNCaP cells, flasks and plates were either pre-coated with poly-D-lysine (BD Biosciences, USA) or coated manually (section 2.1.3).

2.1.3 Coating flasks with poly-D-lysine

Poly-D-lysine hydrobromide (Sigma, USA) was supplied desiccated and 5 mg was dissolved in 50 ml of sterile tissue culture grade water (Sigma). 0.5 ml of solution was added to a 25 cm² (T25) flask and 1.5 ml to a T75, then left at room temperature for 5 minutes before removing the solution and washing the inside of the flask with sterile water. The flasks were dried out before use.

2.1.4 Cell subculture

Subculturing of the cells, as well as experimental work, was carried out in a Class II laminar flow cabinet (Holten, UK) under sterile conditions. Subculturing took place when the cells reached 80-90% confluency. The media was poured off and the cells were washed with 5 ml of 0.01 M phosphate buffered saline (PBS, 13.8 mM NaCl, 2.7 mM KCl, pH 7.4) to remove any traces of FBS. To lift the cells from the base of the flask, 2 ml of 0.5% trypsin ethylene-diamine tetra-acetic acid (EDTA) was

added. Following incubation of the cells at 37°C for five minutes, media was used to inactivate the trypsin and the cells were transferred to a 20 ml tube, which was spun at 1300 rpm for three minutes. The supernatant was removed and the cells were resuspended in 1-4 ml of media. A portion of this cell solution was added to a fresh T75 flask containing 10 ml of media. Cells were kept for twenty passages before reconstitution of a fresh batch of frozen cells.

2.1.5 Counting cells

Cells were trypsinised, spun down and resuspended in media as described above. After vigorous mixing, 10 µl of the cell solution was added to a 1.5 ml tube. Cell viability was tested using a trypan blue dye exclusion assay. 90 µL 0.4% trypan blue solution (w/v) was added to cells. 10 µL of the trypan blue-cell suspension was added to each counting chamber of a haemocytometer (Hausser Scientific, PA, USA). The number of viable cells (unstained as they actively exclude trypan blue dye) in four grids in each chamber was counted. The number of cells/ml was calculated as follows, where the dilution of the grid was 10,000 and the dilution factor was 10:

$$[(\text{Average number of cells per grid}) \times (\text{dilution of grid}) \times (\text{dilution factor})]$$

2.1.6 Making cell stocks

A 70 – 80% confluent T75 flask of low passage cells was trypsinised and spun down in a 20 ml tube. The cells were resuspended in 2250 µl of complete media and 300 µl of FBS was added. 950 µl of the suspension was transferred to each of three 1.5 ml screw-top tubes, then 50 µl of dimethyl sulphoxide (DMSO, Sigma) was added and the tubes were immediately placed at -80°C in a Cryo 1°C Freezing Container (Nalgene Labware, Thermo Fisher Scientific) filled with isopropanol.

2.1.7 Reconstitution of frozen cells

Frozen cell stocks were defrosted by placing the tube briefly in a 37°C water bath and the cells were immediately transferred to a 20ml tube containing 8 ml of complete media and 2 ml of FBS. This was spun at 1300 rpm for 3 minutes and the media was removed. The cell pellet was resuspended in 1 ml of complete media, which was transferred to a T25 flask containing an additional 10 ml of media. The

following day the cells were washed with PBS and given fresh media. When the cells were confluent they were transferred to a T75 flask.

2.1.8 *Mycoplasma testing*

Mycoplasma testing was routinely carried out every three months using the MycoAlert mycoplasma detection kit (Cambex, UK) following the manufacturer's instructions.

2.2 Hypoxia

Cells were grown in an Invivo₂ 400 hypoxia chamber (BioTrace, UK). Levels of oxygen were 0.5% with 5% carbon dioxide. Aerobic conditions are defined as 21% oxygen.

2.3 Irradiation

Cells were irradiated using a RS225 cell irradiator (Xstrahl, UK) at a dose rate of 3.25 Gy/minute. The plates/flasks were placed at the centre of a broad radiation beam to ensure uniform delivery.

2.4 Clonogenic assay

Cells were seeded in a 6-well plate at a concentration of 1000-3500 cells/well depending on the treatment conditions. Following treatment, the plates were incubated at 37°C for 10-14 days to allow colony formation. The media was removed from the plates and replaced with crystal violet (0.05% w/v in methanol (25%)/water (75%)) for 5 minutes. The crystal violet was removed and the wells were washed with water and allowed to dry before counting the number of colonies with greater than 50 cells using a ColCount machine (Oxford Optronix Ltd., U.K.). The surviving fraction was calculated by comparing colony numbers for treated plates to the plating efficiency (number of colonies in untreated wells divided by the number of cells seeded).

2.5 Vector preparation

2.5.1 *Agar plate preparation*

40g of agar was dissolved in 1 litre of deionized water in a sterile flask then autoclaved. The agar was heated to dissolve it then added to 85mm petri dishes along with the antibiotic chloramphenicol at a concentration of 20 µg/ml or

ampicillin at 50 µg/ml. The agar plates were sealed with parafilm and stored at 4°C until needed.

2.5.2 Transformation

Transformation of the vector was carried out using SURE (Stop Unwanted Rearrangement Events) competent cells (Agilent Technologies, Stratagene Products, USA). SOC medium (Sigma) was heated at 42°C. The SURE cells were thawed on ice and 100 µl was added to pre-cooled round-bottom tubes (BD Biosciences), followed by 1.7 µl of β-mercaptoethanol. The tubes were kept on ice for 10 minutes with careful mixing of the cells every two minutes. The desired vector DNA concentrations were prepared and 1 µl was added to the tubes. 1 µl of pUC18 control DNA was added to another tube and the final tube had no DNA (untransformed control). The tubes were mixed and left on ice for 30 minutes, then heat-pulsed for 45 seconds at 42°C in a water bath. They were placed on ice for 2 minutes before adding 0.9 ml of SOC medium to each tube and incubating them at 37°C with shaking (225-250 rpm) for 1 hour. Luria Broth (LB) agar plates, containing the appropriate antibiotic, were warmed to 37°C, inoculated with less than 200 µl of each of the DNA samples and incubated overnight at 37°C.

2.5.3 Growth of bacterial cultures

Following transformation, the agar plates were examined for colony growth. The optimal concentration of DNA gave single colonies which were individually removed using a pipette tip and placed in a 50 ml tube with 3 ml of antibiotic-containing LB broth. This was repeated for nine more colonies. The tubes were shaken at 150 rpm overnight at 37°C.

2.5.4 DNA extraction - Miniprep

DNA was purified from bacteria-containing broth using Miniprep kits (Qiagen) as per the manufacturer's protocol. The DNA was eluted in 50 µl of Buffer EB.

2.5.5 Glycerol stocks

1 ml of broth culture was combined in a 1.5 ml screw-top tube with 179 µl of sterile glycerol to give a concentration of 15% glycerol. The mixture was vortexed and stored at -80°C for future vector preparation.

2.5.6 Growth of bacterial cultures from glycerol stocks

Agar plates were warmed in the incubator for 30 minutes. The glycerol stock of the vector-containing bacteria was defrosted on ice and a sterile loop was used to streak it on the agar plate. The plate was then returned to the incubator and left overnight.

Starter culture

5 ml of sterile terrific broth (47.6 g terrific broth powder (Sigma), 8 ml glycerol, made up to 1 litre with deionised water) was combined with the appropriate antibiotic. Using a pipette tip, a colony of bacteria was removed from the agar plate and placed in the tube. This was repeated nine more times and the tubes were placed at 37°C with shaking for 8 hours.

Overnight culture

10 – 20 µl of the bacterial broth from the starter culture was added to 10 ml of broth containing the appropriate antibiotic. Five tubes were prepared in this manner and left overnight at 37°C with shaking.

2.5.7 DNA extraction - Midiprep

The NucleoBond Xtra Midiprep kit (Macherey-Nagel, Germany) was used to extract DNA following starter and overnight culture preparation as per the manufacturer's protocol. The DNA was then eluted using 600 µl redissolving buffer TRIS (5 mM Tris/HCl, pH 8.5). The DNA yield was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

2.6 Agarose gel electrophoresis

To make a 1% gel, 1g of agarose (Cambrex, ME, USA) was added to 100 ml of 1 × Tris/Acetate/EDTA (TAE) buffer in a glass bottle. The mixture was heated in a microwave for short bursts of time to dissolve the agarose and allowed to cool. 50 ml of agarose was decanted into a tube and 5 µl of SYBR Safe DNA gel stain (Invitrogen) was added. The agarose was cast in a gel plate, placed in the electrophoresis apparatus and covered with 1 × TAE buffer. 5 µl of each DNA sample were mixed with 1 µl of 6 × loading dye and run against the appropriate ladder for approximately 30 minutes at 90 V. The gel was imaged under ultraviolet light using the BioSpectrum Imaging System (UVP).

2.7 CellTiter 96[®] non-radioactive cell proliferation assay (MTT assay)

The CellTiter 96[®] non-radioactive cell proliferation assay (Promega, USA) was used to measure cell number. 15 µl (96-well plate, 200 µl media/well) or 37.5 µl (24-well plate, 500 µl media/well) of dye solution was added to each well of the plate, which was then incubated at 37°C for 4 hours. 100 µl (96-well plate) or 200 µl (24-well plate) of solubilisation solution/stop mix was transferred to each well, followed by a further 1 hour long incubation. The solution was mixed and if a 24-well plate had been used, 250 µl of the contents of each well was moved to a 96-well plate. The absorbance was then read at 570 nm using a spectrometer (Victor Light, Perkin Elmer, CA, USA)

2.7.1 Cell number standard curve

To normalise PSA levels to cell number (concentration per 100,000 cells) following PSA enzyme immunoassay (EIA), a standard curve of cell number vs. absorbance during MTT assay was created (Appendix D). 22Rv1 and LNCaP cells were counted and a range of cell concentrations were seeded in the wells of a 24-well plate. Following incubation for 4 hours to allow adherence of the cells, an MTT assay was carried out. The resulting absorbance values were plotted against the known cell concentrations. The equation of the line was used in subsequent experiments to calculate the final cell concentration after MTT assay.

2.8 Cell viability following pPPE-CD/5-FC treatment

In Chapters 3 and 4 cell viability assays were carried out to assess the effect of pPPE-CD/5-FC treatment in combination with hypoxia (0.5% oxygen) and radiation treatment respectively. 22Rv1 and LNCaP cells were seeded in 96-well plates in 100µl of 10% CD-FBS media at a concentration of 2.5×10^4 cells/well. The following day the cells in four plates were transfected with one of the two vectors pPPE-CD or pCMV-CD or left untransfected. In the fifth plate, 3 wells were transfected with each of the vectors or left untransfected and the last well was transfected with a GFP-expressing vector as a control. The transfection was carried out as described in section 2.10 with modifications for the small volume of a 96-well plate: 0.1 µg of DNA and 0.3 µl of TransFast transfection reagent were used and this was made up to 40 µl with serum free media. In the case of the untransfected controls, 40 µl of serum free media was added.

An MTT assay was carried out on the cells in plate 5 (section 2.7). This assay gave a measurement of cell number following transfection but before treatment of the cells. The media was replaced with fresh CD-FBS media in air or in the hypoxia chamber and 1000 nM DHT was added to half the wells. Two hours after DHT addition the plates were treated with 2 Gy of radiation or mock irradiated. After three days, the media in all the wells was replaced with fresh CD-FBS media. Three concentrations of 5-FC (0.1, 1, 5 mM) were added to the wells. The plates were kept in the incubator for a further three days, before an MTT assay was carried out on the cells to assess their viability.

2.9 PSA-EIA

A CanAg PSA EIA kit (Fujirebio Diagnostics, Inc, PA, USA) was used to determine the PSA protein concentration in media taken from treated cells. Media was stored at -20°C until needed. Prior to assay, the wash concentrate was diluted 1:25 with deionised water. To make the antibody solution 50 µl of Tracer, HRP-Anti PSA was added to 1 ml of Biotin Anti-PSA per strip of eight wells. The strips were placed in the frame provided and were washed with the wash solution by filling each well to the top and then completely removing the solution. 25 µl each of the PSA calibrators, controls and samples were added to the wells in duplicate, followed by 100 µl of antibody solution. The plate was placed on a shaker for one hour at room temperature then washed six times. 100 µl of TMB (3,3',5,5'-tetramethylbenzidine) HRP-substrate was added to each well and the samples were incubated on a shaker for 30 minutes at room temperature. 100 µl of Stop Solution was pipetted into the wells and the absorbance was read at 450 nm. The readings for the PSA calibrators were used to make a standard curve, from which the PSA concentrations of the samples could be calculated.

2.10 Transfection

TransFast Reagent stock solution was prepared the day before transfection. The reagent (1.2 mg) was allowed to reach room temperature before adding 400 µl of nuclease-free water, followed by vortexing for 10 seconds and storage at -20°C. The following day, DNA for transfection was mixed with serum-free antibiotic-free media and vortexed. TransFast was vortexed and added to the DNA/media mixture, which was vortexed again, then left at room temperature for 15 minutes. The media

was removed from the cells and the DNA/media was vortexed and added to the cells, followed by incubation for 3 hours in a humidified incubator at 37°C with 5% CO₂. The media was then replaced with complete media. A GFP expressing vector was used as a positive control and the following day the cells were assessed under a fluorescent microscope to check if the transfection had been successful.

2.11 Protein collection

Before protein collection, all lysis buffer components were allowed to thaw on ice. Cells for protein collection were washed with cold PBS (4°C). 3 ml of PBS was added to the flask and the cells were detached from the surface using a cell scraper (Starstedt). The cell-containing PBS was transferred to a 20 ml tube on ice and spun in a centrifuge at 1300 rpm for 3 minutes at 4°C. For hypoxic samples these steps were carried out in the hypoxia chamber before removing the tubes to spin them at 1300 rpm. Per sample, 40 µl of Radio Immuno Precipitation Assay (RIPA) buffer, 2 µl of phenylmethylsulfonyl fluoride (PMSF) protease inhibitor, 2 µl of sodium orthovanadate and 4 µl of protease inhibitor were mixed in a chilled 1.5 ml tube. The PBS was removed from the tubes by aspiration and each cell pellet was resuspended in 30 µl of the RIPA solution, transferred to a chilled 1.5 ml tube and left on ice for 15 minutes with vortexing every few minutes. The lysed cells were then spun at 13,500 rpm for 15 minutes at 4°C before transferring the protein-containing supernatant to a chilled tube which was stored at -80°C until needed.

2.12 Bradford assay

A Bradford assay was used to quantify protein concentration following extraction. Preparation of the standards and samples was carried out on ice. A 0.1 mg/ml stock of bovine serum albumin (BSA) was prepared by diluting 10 µl of 10 mg/ml BSA (Promega, WI, USA) in 990 µl of deionized water. A set of standards was made up by combining different amounts of this BSA solution with deionized water as follows:

Table 2.1: Composition of standards for Bradford Assay

<i>Final concentration of BSA ($\mu\text{g/ml}$)</i>	<i>0.1 mg/ml stock (μl)</i>	<i>Deionized water (μl)</i>
0	0	500
1	5	495
2	10	490
4	20	480
8	40	460
16	80	420
32	160	340

1 μl of each protein sample was combined with 500 μl of water in a 1.5 ml tube. 500 μl of Bradford reagent was added to the tubes of standards and samples. 200 μl from each tube was added to a 96-well plate and the absorbance was read in a spectrophotometer at 595 nm. Readings were corrected for background absorbance by subtracting the absorbance of the 0 $\mu\text{g/ml}$ standard from the other standards and samples. Absorbance of the standards versus their protein concentrations was plotted using Microsoft Excel and the equation of the trend line was used to calculate the protein concentration of the samples.

2.13 Western blotting

2.13.1 Polyacrylamide gel electrophoresis

Fresh 10% (w/v) ammonium persulfate (APS) was prepared by diluting 0.06 g in 600 μl deionized water. Depending on the size of the protein of interest, resolving gels of 8 – 12% were prepared using reagents described in Table 2.2. The resolving gel was pipetted between the glass plates of a minigel apparatus and isopropanol was added on top to prevent evaporation. The gel was allowed to set for approximately 15 minutes before adding the stacking gel. This consisted of 1.525 ml of deionized water, 333.5 μl 30% acrylamide:bisacrylamide (37.5:1), 635 μl 0.5 M Tris HCl, pH 6.8, 25 μl 10% (w/v) SDS, 25 μl 10% APS and 2.5 μl TEMED per gel.

Table 2.2: Reagents in resolving gel

	8% gel	10% gel	12% gel
Deionized H ₂ O	2.35 ml	2.05 ml	1.7 ml
Acrylamide	1.35 ml	1.65 ml	2 ml
1.5M Tris, pH 8.8	1.25 ml	1.25 ml	1.25 ml
10% SDS	50 µl	50 µl	50 µl
10% APS	37.5 µl	37.5 µl	37.5 µl
TEMED	7.5 µl	7.5 µl	7.5 µl

Protein samples were made up to 10 µl using deionized water and kept on ice. 10 µL of 2× Laemmli buffer (2 ml Tris HCl pH 6.8, 5 ml 10% (w/v) SDS, 1 ml 2-β-Mercaptoethanol, 2 ml glycerol, 0.05g (w/v) bromophenol blue) was added to each sample, and protein samples were subsequently denatured at 95°C for 5 minutes.

The polyacrylamide gel was placed in a gel rig and covered in 1× SDS-PAGE running buffer. 10 µL of Precision Plus Protein Dual Colour Standards (Bio-Rad Laboratories, CA, USA) was run alongside the 20 µL protein samples at 200 V for 30 – 60 minutes.

2.13.2 Transfer

The proteins were transferred onto biotrace polyvinylidene flouride (PVDF) membrane (GE Healthcare Ltd., Buckinghamshire U.K.) which had previously been activated in 100% methanol for 1 minute. Membranes and gels were soaked in cold 1× transfer buffer for 12 minutes then placed in the transfer rig, covered with transfer buffer and transferred at 100 V for 1 hour.

2.13.3 Blotting

Following protein transfer, the PVDF membrane was blocked with 5% (w/v) non-fat milk (Marvel) in Tween-PBS for 1 hour at room temperature, then washed four times with Tween-PBS for five minutes per wash. The primary antibody was diluted

in milk or bovine serum albumin (BSA), the concentration depending on the particular antibody, and added to the membrane. The membrane was incubated overnight at 4°C.

The following day, the wash steps were repeated. The secondary antibody (HRP-conjugated) was diluted in milk (1:2000) and incubated with the membrane for one hour at room temperature, followed by further wash steps. The presence of bound antibody complexes was detected using the Supersignal West Pico Chemiluminescent substrate kit (Thermo Scientific, USA). X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan) was placed in the cassette with the membranes in a dark room. The film was developed using a medical film processor (Agfa-Gevaert, Mortsel, Belgium).

Table 2.3: Antibodies used in Western Blotting

Primary antibody	Concentration	Manufacturer	Secondary antibody	Concentration	Manufacturer
Cytosine deaminase	1:1200	mtm laboratories	HRP rabbit anti-mouse	1:2000	DakoCytomation
Androgen receptor	1:250	Santa Cruz	HRP rabbit anti-mouse	1:2000	DakoCytomation
NFκB p50	1:500	Santa Cruz	HRP rabbit anti-goat	1:2000	Sigma
HIF-1α	1:500	Cell Signalling	HRP goat anti-rabbit	1:2000	

2.13.4 Loading control blotting

The membranes were probed for a second protein to ensure the concentration of protein loaded in each well was the same. One of two primary antibodies was used, β-actin (1:5000 in milk) or α/β-tubulin (1:1000 in 5% BSA). The membrane was incubated with the primary antibody for one hour at room temperature then washed. The secondary antibody was diluted in milk (1:2000), HRP rabbit anti-mouse for β-actin, HRP goat-anti rabbit for α/β-tubulin. The membrane was incubated with the secondary antibody for one hour at room temperature then washed. The presence of bound antibody complexes was determined as described (section 2.13.3).

2.14 TaqMan gene expression assay

2.14.1 RNA extraction - RNeasy mini kit

Following treatment, the cells were trypsinized and counted. Pellets of 3-4 million cells were frozen at -80°C until needed. RNA extraction was carried out using the Qiagen RNeasy mini kit.

The cell pellet was defrosted and disrupted by flicking the tube. 350 µl of Buffer RLT was added to the cells, which were vortexed. Cells were homogenized by adding the lysate to a QIAshredder spin column in a 2 ml collection tube and spinning the tubes in a centrifuge at full speed for 2 min. 350 µl of 70% ethanol was added to the lysate and the samples were pipetted to mix. Samples were then transferred to an RNeasy spin column in a 2 ml collection tube and spun at $\geq 10,000$ rpm for 15 seconds.

700 µl of Buffer RW1 was added to the spin columns and they were spun at $\geq 10,000$ rpm for 15 seconds. The flow-through was discarded and 500 µl of Buffer RPE was added and spun at $\geq 10,000$ rpm for 15 seconds. This step was repeated with another 500 µl of Buffer RPE, this time spinning for 2 min at $\geq 10,000$ rpm. To prevent ethanol carry-over the spin column was placed in a fresh collection tube and spun at full speed for 1 min. The column was put into a 1.5 ml tube. 30 µl of RNase free water was added and the samples were spun at $\geq 10,000$ rpm for 1 minute. To increase the RNA yield this step was repeated using another 30 µl of RNase free water. A Nanodrop 1000 spectrophotometer was used to measure the concentration and purity of the RNA in each sample. RNA was stored at -80°C until needed.

2.14.2 RNA extraction – TRI Reagent

TRI Reagent (Sigma) was used to isolate RNA. Media was removed from the flasks and 1ml of TRI Reagent was added to each one. A cell scraper (Sarstedt) was used to remove the cells from the flask surface and the TRI Reagent/cell mixture was transferred to a 1.5 ml tube. At this point the samples could be stored at -80°C for extraction at a later date (up to two months). Before proceeding, samples were kept at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. 100 µl of bromo-3-chloropropane was added to each sample, followed by vortexing for 15 seconds. Samples were kept at room temperature for 15 minutes and then

centrifuged at 13,500 rpm at 4°C for 15 minutes. This resulted in separation into a lower phenol-chloroform phase, an interphase that contains the DNA and proteins and an upper aqueous phase containing the RNA. This aqueous phase was transferred to a fresh tube and 500 µl of isopropanol was added to each sample to precipitate the RNA. After incubation at room temperature for 10 minutes, the tubes were centrifuged at 13,500 rpm at 4°C for 8 minutes.

The supernatant was removed and 75% ethanol was added to wash the pellet. The samples were then spun at 13,500 rpm at 4°C for 8 minutes, the ethanol was removed and the pellet was allowed to dry in the air for 5 minutes. The RNA was then resuspended in 30 µl DNase/RNase free water and the concentration was measured using a Nanodrop 1000 spectrophotometer.

2.14.3 Tissue samples

In Chapter 3, qRT-PCR analysis was carried out using RNA extracted from prostate tissue specimens of men who had undergone radical prostatectomy for primary prostate cancer. These samples were obtained through the histopathology archives dating from 2000 in St James's Hospital. Suitable formalin fixed paraffin embedded (FFPE) blocks of each case were selected and corresponding Haematoxylin and Eosin (H&E) stained sections were reviewed and annotated by a pathologist, Dr. Ciara Barrett.

11 cases of prostate cancer and matched histologically benign tissue were used for nucleic acid isolation by researchers in the Prostate Molecular Oncology Research group. A series of 5µm sections were cut from the FFPE blocks. The first and last sections were H&E stained and compared with the pathologically evaluated slides. Intervening sections were deparaffinized; tissue was scraped from within the marked target areas and total RNA was extracted using a RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion Inc, Austin, TX) and quantified using a Nanodrop-1000 Spectrophotometer.

2.14.4 DNase treatment

RNA was treated with DNase (Ambion) to remove possible DNA contamination. The RNA was transferred to a 0.5 ml tube and 0.1 volume of 10x DNase I buffer and 1 µl of rDNase I were added. The samples were incubated in a thermal cycler

(MJ Research PTC-200, Bio Sciences) at 37°C for 30 minutes. 0.1 volume (at least 2 µl) of DNase inactivation reagent was added to the samples, which were then incubated at room temperature for 2 minutes with mixing. The tubes were centrifuged at 10,000 g (10,400 rpm) for 1.5 minutes and the RNA was moved to a fresh tube, leaving behind the DNase inactivation reagent.

2.14.5 cDNA synthesis

Following RNA extraction, reverse transcription was carried out to produce cDNA for real-time PCR using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The RNA samples were added to a strip tube and then made up to a final volume of 10 µl using nuclease free water. 2× reverse transcription reaction master-mix was made up on ice. For each reaction, the components were added in the following concentrations: 2 µl 10× reverse-transcription reaction buffer, 0.8 µl 100mM dNTP mix, 2 µl 10× reverse transcription random primers, 1 µl multiscribe reverse transcriptase, 4.2 µl nuclease free water. 10 µl of the master-mix was added to each sample tube in the strip, pipetting to mix. The tubes were placed in an automated DNA thermal cycler. The cycle conditions consisted of 10 minutes at 25°C followed by 2 hours at 37°C, then 5 seconds at 85°C. The resulting cDNA was stored at -20°C until needed.

2.14.6 qRT-PCR

A PCR reaction master mix of either 10 or 20 µl per sample was made up on ice, which contained the following components per reaction: 0.5/1 µl 20× TaqMan Gene Expression Assay buffer, 5/10 µl TaqMan Gene Expression Master Mix and 3.5/8 µl RNase free water. 9/19 µl of the master mix was added to the required number of wells of a 96 or 384-well MicroAmp Optical reaction plate (Applied Biosystems). 1 µl of the cDNA samples was then added to the wells of the plate. Each sample was run in triplicate or quadruplicate. The plate was sealed and spun at 1000 rpm for 30 seconds, then placed in a 7500 (96-well plates) or 7900HT (384-well plates) Sequence Detection System (Applied Biosystems) and run at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Table 2.4: Gene Expression Assays

Gene	Applied Biosystems code
<i>HIF-1A</i>	Hs00936371_m1
<i>GSTP1</i>	
<i>AR</i>	Hs00907244_m1
<i>PSA</i>	Hs02576345_m1
<i>FKBP51</i>	Hs01561006_m1

2.14.7 Data analysis

The $\Delta\Delta C_T$ method was used to calculate the relative quantity of gene expression. The C_T value is the cycle number of the PCR at which the amount of the amplified sample crosses a threshold level. The ΔC_T value is calculated by subtracting the average C_T of the endogenous control from that of the target gene. The $\Delta\Delta C_T$ is then found by subtracting the ΔC_T of the calibrator sample (normally an untreated control) from the ΔC_T of each of the other samples. The relative quantity of target gene expression compared to the calibrator is calculated using the formula $2^{-\Delta\Delta C_T}$.

Firstly, an assay was set up to check if the C_T values for the endogenous control gene were consistent between the various cell lines and under the different treatment conditions. To validate that the comparative C_T method was suitable, the efficiency of the endogenous control amplification was compared to that of the target gene. This was carried out by diluting the cDNA of one of the samples over a five log scale. The log of the amount of cDNA used was plotted against the ΔC_T value for the two genes. The slope of the line should be between -0.1 and 0.1. The relative quantification qRT-PCR experiment was then carried out. Data analysis was carried out using the 700 Software v2.0.1 and Microsoft Excel.

In Chapter 3, the freely available statistical programme Normfinder¹⁹¹ was used to rate the suitability of genes as endogenous controls. Normfinder allows assessment of both inter- and intragroup variability and ranks the genes accordingly to give a measure of stability. For example, the C_t variation within the air/hypoxia sample groups are assessed for each gene as well as the variation between air and hypoxia groups. The lower the value assigned to the gene, the less it varies within/between groups and the better an endogenous control it is. Applied Biosystems 7500 Software v2.01 and RQ Manager v1.2 were used to analyse the results in this chapter.

2.14.8 Human endogenous control plate

In Chapter 3, human endogenous control array cards (Applied Biosystems) were used to assess the performances of 16 commercial endogenous control genes for qRT-PCR. 240 ng of cDNA was used for each sample. The cDNA was made up to 60 μ l using nuclease free water in a 1.5 ml eppendorf tube and 60 μ l of TaqMan Gene Expression Master Mix (Applied Biosystems) was added giving a final cDNA concentration of 2 ng/ μ l. The sample was vortexed and centrifuged. The array card was allowed to reach room temperature for 15 minutes. 100 μ l of the cDNA/master mix was slowly loaded into each of the fill ports. The card was then centrifuged at 1200 rpm for two sets of one minute, then sealed using the TaqMan Array Micro Fluidic Card Sealer (Applied Biosystems). The qRT-PCR run was set up and performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems).

2.15 siRNA

siRNA knockdown of *HIF-1A* was carried out using the N-TER Nanoparticle siRNA transfection system (Sigma) and the pre-designed siRNA against *HIF-1A* (MISSION siRNA, SASI_Hs02_00332063, Sigma). 22Rv1 and LNCaP cells were seeded in 6-well plates at 2.5×10^5 cells/well. 16 hours later the nanoparticle formation solution was prepared. The siRNA was diluted in TE buffer to a concentration of 5 μ M and placed on ice. The appropriate amounts of siRNA dilution buffer and siRNA were added to 1.5 ml tubes and vortexed. In separate tubes the N-TER peptide was diluted with water and vortexed. The contents of the tubes were then combined to make the Nanoparticle Formation Solution (NFS). A control NFS was made up that did not contain siRNA or N-TER peptide. The tubes were stored at room temperature for 15-20 minutes. The NFS was diluted in serum free RPMI media to obtain the desired concentration of siRNA. 1500 μ l was added to each well, followed by 1500 μ l of 2 $\times$ serum-containing media to give a final siRNA concentration of 10-30 nM. Following 24-48 hour incubation, RNA was collected from the samples and qRT-PCR was used to assess the success of the siRNA knockdown.

2.16 Creation and characterisation of androgen receptor overexpressing 22Rv1

In Chapter 6, the cell line 22Rv1 was stably transfected with an AR-expressing vector with the aim of creating an improved *in vitro* model of primary prostate cancer in which AR levels are high.

2.16.1 The vector

The vector pcDNA3.1-AR expresses AR under the control of the CMV promoter. It also contains the gene for resistance to the drug Geneticin (neomycin). pcDNA3.1-AR was obtained from Prof. Visakorpi, Institute of Medical Technology, University of Tampere, Finland.

2.16.2 Transformation/ DNA extraction

Transformation of pcDNA3.1-AR was carried out using SURE competent cells (Agilent Technologies - Stratagene Products, USA) as described in section 2.5.2. Various concentrations of the pcDNA3.1-AR DNA were prepared and 1 µl of each was added to a tube. After streaking the agar plates and leaving overnight at 37°C, suitable single colonies were selected. 10 colonies were grown in LB broth overnight and a Miniprep was carried out to isolate the vector DNA (section 2.5.4)

2.16.3 Agarose gel electrophoresis

Agarose gel electrophoresis (section 2.6) was carried out to assess whether the plasmid had been successfully isolated by the Miniprep procedure. 1 µl of DNA was mixed with 4 µl of DNase free water and 1 µl of 6 x loading dye and run against a 1 Kb ladder.

2.16.4 Restriction digest

A restriction digest was carried out to ensure the plasmid had the expected sequence and size. The restriction enzymes HindIII and BglII were used. 5 µl of DNA, 2 µl of 10 × buffer, 12.5 µl of DNase free water and 0.5 µl of the restriction enzyme were combined in an 1.5 ml tube and heated for 1 hour at 37°C. The samples were run on a 1% agarose gel. Uncut DNA was also run on the gel as a control.

2.16.5 Glycerol stocks

Following the positive result from the agarose gel electrophoresis, glycerol stocks of pcDNA3.1-AR were prepared using the bacteria-containing LB broth that was

grown after transformation of the vector. Glycerol stock preparation was carried out as described in section 2.5.5.

2.16.6 DNA extraction- Midiprep

A starter and overnight culture were set up (section 2.5.6) using colonies of the AR-expressing vector from the agar plates. A Midiprep (section 2.5.7) was used to isolate the DNA.

2.16.7 Optimisation of Geneticin concentration

The optimal concentration of the antibiotic Geneticin (neomycin) that would kill 90-100% of normal 22Rv1 cells was determined. 22Rv1 cells were seeded in 6-well plates at a concentration of 0.2×10^5 in 2 ml of complete media. Two days later a range of concentrations of Geneticin were added to the wells, three wells per concentration. The concentrations were 0 $\mu\text{g}/\mu\text{l}$, 50 $\mu\text{g}/\mu\text{l}$, 100 $\mu\text{g}/\mu\text{l}$, 200 $\mu\text{g}/\mu\text{l}$, 400 $\mu\text{g}/\mu\text{l}$, 600 $\mu\text{g}/\mu\text{l}$ and 800 $\mu\text{g}/\mu\text{l}$. The media was changed every 4/5 days and fresh Geneticin was added. Two weeks later the cell number in each well was determined using the trypan blue exclusion assay described in section 2.1.5. The average results for the replicates were used to determine the optimal concentration of Geneticin to use in future experiments.

2.16.8 Transfection

Transient transfection of 22Rv1 cells with pcDNA3.1-AR was carried out in T25 flasks using TransFast Transfection Reagent (section 2.10). A GFP containing vector was used as a positive control for the transfection and a flask of untransfected cells was used as a negative control. Four days later the pcDNA3.1-AR transfected and untransfected cells were counted and seeded in T75 flasks. The untransfected cells acted as a positive control for cell kill by Geneticin. Geneticin was added at a concentration of 400 $\mu\text{g}/\mu\text{l}$. The Geneticin-containing media was changed every 5-7 days with subculturing to ensure all cells that had been killed were removed.

2.16.9 Cell cloning by serial dilution

Once the pcDNA3.1-AR transfected 22Rv1 cells had been established, cell cloning was carried out to ensure the cell line was derived from a single cell. Serial dilution in a 96-well plate was used. A suspension of 2×10^4 cells/ml was made up and 200 μl (4000 cells) was added to well A1. 100 μl of complete media was added to the

other wells in the first column. 100 μ l of the cell suspension was transferred from column A1 to column B1 and gently mixed. This dilution was repeated for the other wells in the column (100 μ l from B1 to C1, and so on) and 100 μ l was discarded from the last well (H1). Then, using a multi-channel pipette, 100 μ l of complete media was added to each of the wells of the first column, with mixing. With the same pipette, 100 μ l was then transferred from the wells of the first column to the wells of the second column. This was repeated across the 96-well plate, discarding 100 μ l from the last column. 100 μ l of complete media was added to each well in the plate, which was then incubated at 37°C. The serial dilution was repeated for a number of other concentrations of the starting cells suspension: 1×10^4 cells/ml, 0.5×10^4 cells/ml and 0.25×10^4 cells/ml.

The flasks were examined to identify wells containing single colonies. Four wells were chosen and the cells were transferred to a 24-well plate 9-14 days after the serial dilution was carried out. Approximately 3 weeks later cells were established in one of the wells of the 24-well plate and could be transferred to a T25 flask. Three days later the media in the flask was replaced with Geneticin-containing media (400 μ g/ μ l) to ensure the cells were expressing the vector.

The entire serial dilution procedure was then repeated to increase the probability that the cell line was established from a single cell. An age-matched control 22Rv1 cell line (22Rv1-AMC) was also developed which was maintained in complete media for an equivalent amount of time to the stably transfected 22Rv1 cells.

2.16.10 Calpastatin and Ionomycin treatment (new section)

In Chapter 6, cells were treated with the calpain inhibitor calpastatin (Sigma) or the calpain activator ionomycin (Cell Signaling Technology) to assess the influence of calpains on the levels of full length and truncated AR. 22Rv1, 22Rv1-ARhi and LNCaP cells were seeded in T25 flasks (Western blotting) or 24-well plates (PSA EIAs) in CD-FBS and incubated for 72 hours. The media was then supplemented with 1000 nM DHT and the cells were treated with calpastatin or ionomycin or treated as controls. 0.5 mg of calpastatin was dissolved in water and the cells were treated to give a final concentration of 1 μ M. 2.5 mg of ionomycin was dissolved in DMSO and cells were treated for 10 minutes with 10 μ M or the equivalent amount of DMSO before replacing the media with fresh DHT-containing CD-FBS media.

Half the plates/flasks were placed in hypoxia (0.5% oxygen), with the other half incubated under aerobic conditions (21%). After 72 hours protein was collected for Western blotting or MTT assay and media collection was carried out for subsequent PSA EIA.

2.17 Bicalutamide treatment

In Chapter 6, the response of 22Rv1 and 22Rv1-ARhi to the anti-androgen bicalutamide was compared using MTT and clonogenic assays.

2.17.1 MTT assay

22Rv1, 22Rv1-ARhi and 22Rv1-AMC cells were seeded, 5000 cells/well, in a 96-well plate in CD-FBS media. After 48 hours the cells were treated with 1000 nM DHT in combination with 0, 1, 10 or 50 μ M bicalutamide under conditions of 21% and 0.5% oxygen. Ethanol with increasing amounts of DMSO was used as a negative control for DHT and bicalutamide respectively. After a further 4 days, an MTT assay was carried out (section 2.7) and the number of viable cells/well was elucidated.

2.17.2 Clonogenic assay

22Rv1 and 22Rv1-ARhi cells were seeded in 6-well plates in 2 ml CD-FBS media at a concentration of 1000 cells/well. After 48 hours the cells were treated with 1000 nM DHT with and without bicalutamide (10 or 50 μ M) or with ethanol and DMSO as negative controls. After 4 days of treatment the media was replaced with complete media. 10-14 days later the plates were stained with crystal violet and the colonies were counted as described in section 2.4

2.18 Spheroids

In Chapter 7, a novel technique was used to develop a 22Rv1 spheroid model to try to improve on current *in vitro* cell culture methods. This involved the use of an advanced suspension media, Happy Cell (HC), which was developed by Biocroí Ltd.

2.18.1 Cell culture

The HC media contained Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) FBS and 1% Penicillin/streptomycin and an additional additive. Following counting, the cells were added to the HC media at the

desired concentration and resuspended gently to get an even distribution throughout the media. An incubation time of 48-72 hours resulted in spheroid formation. As a control, adherent 22Rv1 cells were grown in normal DMEM without the extra additive.

2.18.2 Confocal microscopy

22Rv1 cells were seeded in an 8-chambered Lab-Tek slide (Thermo Scientific) at a concentration of 1×10^5 cells/well in 500 μ l HC media and incubated at 37°C for 2 days to allow spheroid formation. 25 μ l Hoechst 33342 dye (1 mg/ml, Sigma Aldrich) was added to each well. Propidium iodide (PI) was diluted 1:100 with PBS and 50 μ l was added to the wells. An Axiovert 200M laser scanning confocal microscope (Zeiss) was used to image the cells following staining. The correct lasers were selected to allow fluorescent imaging of Hoechst (excitation 355 nm, emission 465 nm) and P.I. (excitation 536 nm, emission 617 nm). Single images were obtained, as well as a Z-stack of images (cross-section through a spheroid by taking multiple pictures a set interval apart).

2.18.3 Automated liquid handling

Two different automatic liquid handling systems were used to dispense cell-containing media.

Echo

22Rv1 cells were counted and a 300 μ l cell suspension of 4×10^6 cells/ml was prepared in HC media and DMEM media. 5 μ l of Hoechst dye was added to each. The Echo Liquid Handling System (Labcyte) uses sound waves to dispense set amounts (2.5 to 10,000 nl) of liquid onto a plate surface. The cell-containing media was gently mixed before 40 μ l was placed in the source well of the Echo and used to dispense 100 nl into 24 wells of a 384-well plate at various time points (1, 5, 10 and 20 minutes). The number of cells in each well was counted using the IN Cell Analyzer 1000 high-content screening system and compared between HC media and normal DMEM media.

Deerac

A Deerac Fluidics Equator Eight-Tip Pipetting System (Promega) was used to dispense 200 nl of cell-containing HC media onto 'Nanowell' slides (plastic slides

with wells that hold up to 400 nl of liquid). The wells were imaged after seeding and then again on subsequent days using the Cellavista Analyzer (Roche)

2.18.4 PSA EIA

22Rv1 cells were seeded in 24-well plates, 0.9×10^5 cells/well in 1 ml of media. Normal plates and DMEM media were used for adherent cells; suspension plates and HC media were used for spheroids. 3 days later, the media in half of the wells containing adherent cells was replaced with HC media. 200 μ l of media was added to the wells, either containing DHT to give a final concentration of 1000 nM or with no DHT. After a further 3 days, the spheroid containing media was transferred to 1.5 ml tubes, and spun down to pellet the cells. The media was removed and stored for later PSA EIA. 100 μ l of trypsin was added to the spheroids to break them up and allow MTT analysis of cell number for normalisation of PSA EIA results. 400 μ l of DMEM media was added to the cells and they were transferred to a 24-well plate. As a control, the adherent cells were also trypsinised before the MTT assay. For these cells, the media was removed and stored in 1.5 ml tubes. The cells were washed with PBS, then 100 μ l of trypsin was added to the cells and subsequently deactivated with DMEM media. 37.5 μ l of MTT dye was added to all the wells and the assay was carried out as previously described (section 2.7).

2.18.5 GFP transfection

22Rv1 cells were seeded at a concentration of 1×10^6 cells in a T25 flask in DMEM media. The following day the cells were transfected with a GFP-expressing vector (section 2.10). After a further 24 hours, the cells were counted and seeded in 96-well plates (normal and suspension), 0.9×10^4 cells/well. Over the next week, the adherent cells and spheroids were imaged using an Olympus CKX41 fluorescent microscope and DP70 camera to track GFP expression.

2.18.6 pPPE-CD/5-FC toxicity

22Rv1 cells were seeded in T25 flasks and transfected with pPPE-CD or pCMV-CD, or mock transfected. The following day the cells were seeded in DMEM media in four normal 96-well plates and in HC media in four suspension plates at a concentration of 0.9×10^4 cells/well. The cells were incubated for 72 hours to allow spheroid formation. DHT or ethanol (negative control) was added to the wells by adding 20 μ l of the appropriate media containing DHT or ethanol to give a final

concentration of 1000 nM. The plates were placed in the incubator or hypoxia chamber and half the plates were treated with 2 Gy radiation two hours later. After a further 3 days the cells were treated with 5-FC by adding a further 20 µl of media to obtain a concentration of 1 mM 5-FC and then incubated in air for 72 hours. The cell toxicity was analysed by staining with Hoechst and imaging on a IN Cell Analyzer.

2.18.7 High content screening

IN Cell Analyzer

High-content screening was used in the analysis of the spheroid models of prostate cancer as well as adherent 22Rv1. The cells were imaged live using the fluorescent dye Hoechst 33342 (1mg/ml) which was added to the media 30 minutes before screening. The InCell Analyzer 1000 (GE Healthcare, NJ, USA) automated system for image capture was used to acquire images of multiple fields in each well at 4× magnification. A brightfield image was obtained as well as an image at 360nm excitation and 460nm emission filters for Hoechst fluorescence. The images were captured using a high-resolution charge-coupled device (CCD) camera and subsequently analysed using the IN Cell Analyzer 1000 software. For the spheroids, 20 µl Hoechst 33342 dye (1 mg/ml) was added to each well and for the adherent 22Rv1 cells, 1 µl of dye was used. Both cell cultures were incubated with the stains for 30 minutes.

Cellavista Analyzer

The nanowell plates, as well as some 96-well plates were imaged using the Cellavista Analyzer (Roche), an automated imaging system with brightfield and fluorescence imaging capabilities.

Chapter 3

Gene expression analysis in prostate
cancer: the importance of the endogenous
control

Chapter 3 Gene expression analysis in prostate cancer: the importance of the endogenous control

3.1 Introduction

Quantitative reverse transcriptase PCR (qRT-PCR) is a commonly used laboratory technique in cancer research, enabling the sensitive quantification of gene expression levels.¹⁹² However, it holds a number of potential pitfalls, one of which is normalisation of gene expression results. This is of paramount importance, as there are multiple factors related to the processes of RNA extraction, reverse transcription and qRT-PCR that can introduce variation between samples. These include the source and amount of starting material, RNA integrity, the method of RNA extraction and whether a DNase step is performed.^{191, 193} Several different normalisation methods exist. Using a similar sample size of tissue or cells from *in vitro* cell culture is helpful but not sufficient.¹⁹⁴ Standardising the quantity of total RNA between samples ensures that equal amounts of RNA are used for reverse transcription (the quality of the RNA can also be assessed at this stage) but does not account for potential variability in the downstream qRT-PCR step, such as differences in reverse transcriptase efficiency between individual reactions.

The most commonly used method of normalisation involves using an endogenous control or ‘house-keeping’ gene (HKG), whose expression does not vary between samples and is independent of the pathological state or exposure to environmental stresses or changes in growth conditions. This technique controls for variation in all stages of the procedure up to and including the qRT-PCR measurement. Traditionally this has involved the use of a number of genes such as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) or β -actin (*ACTB*) and the assumption of their constant expression. Huggett *et al.* suggest that this stems from the traditional use of these genes as controls for experiments like Northern blots.¹⁹⁴ However, it is well established that the expression of these genes can vary greatly and may be affected by different treatment conditions.¹⁹⁴⁻¹⁹⁶ So, while certain classical HKGs are often employed to normalize qRT-PCR experiments, it is imperative that their steady-state level of expression is carefully validated under each experimental condition being tested, (i.e. disease phenotype or drug exposure etc.) If this step is not carried out, it can lead to selection of a gene whose fluctuation in expression

will mask small changes in the expression of the target genes being quantified. If the control gene is even moderately affected by the different experimental conditions being studied, it can result in erroneous findings, not necessarily obvious to the researcher.

Aberrant gene expression is a prominent feature of prostate cancer, with overexpression of genes such as *AMACR*, *PCA3* and loss or down-regulation of other genes including *GSTP1*. Whole-genome transcriptome studies have revealed stark differences in expression profiles between tumour and benign prostate and between different stages of prostate carcinogenesis.¹⁹⁷ Validation of these findings and moreover, functional studies on individual genes, largely utilize qRT-PCR. There are a number of confounding factors in relation to prostate cancer that can affect the suitability of potential HKGs, one of which is the tumour microenvironment. In this regard, hypoxia is of particular relevance to this project.

This research project involved the analysis and comparison of gene expression in cell lines grown under aerobic (21% oxygen) and hypoxic (0.5% oxygen) conditions. Before proceeding with these qRT-PCR experiments, careful selection of a suitable endogenous control gene was necessary. HIF-1 is thought to regulate up to 5% of all genes in one or more cell types in response to hypoxia⁴³ and this may include potential endogenous control genes. The issue is complicated by the fact that there is great heterogeneity in gene regulation between different cell lines even if they originate from the same tissue type. Therefore a control gene must be validated for each new cell line even if the treatment conditions remain the same.

In this study, the performance of 16 commercially available endogenous control genes was tested in two prostate cancer cell lines (LNCaP and 22Rv1) cultured with both 0.5% and 21% oxygen. Statistical analysis revealed wide variation in expression for many of the endogenous controls. *In silico* analysis of the regulatory regions of these genes revealed putative HREs in several of them. Four genes were selected for further measurements in a broader panel of cell lines and in normal and cancerous prostate tissue specimens. In this way a suitable endogenous control gene was selected for future work involving the analysis of aerobic and hypoxic samples.

3.1.1 Aims and Objectives

From preliminary qRT-PCR experiments with cell samples grown under 0.5% and 21% oxygen it became clear that it would be necessary to carefully chose the endogenous control gene used for this work, as hypoxia can affect the expression of many of the commonly used control genes.

Aim: To test a panel of potential endogenous control genes for their suitability for aerobic and hypoxic samples from two prostate cancer cell lines.

Aim: To select of a number of candidate genes and test these in four additional cell lines.

Aim: To assess the impact of gene selection on gene expression results.

Aim: To examine the performance of selected genes in tumour and normal tissue samples.

3.2 Results

3.2.1 Optimal endogenous control genes for aerobic (21% oxygen) and hypoxic (0.5%) samples

The performances of 16 commercial endogenous control genes for qRT-PCR were tested in two prostate cancer cell lines, 22Rv1 and LNCaP (Table 3.1). The cell lines were grown in air (21% oxygen) and hypoxia (0.5%) for 24 hours before RNA extraction. The raw C_t values for the two treatment groups are given in Table 3.2. The results for cells grown under aerobic conditions were analysed using Normfinder and the genes were ranked based on their variability (Table 3.3). The C_t data were further analysed to test the variability of the endogenous control genes under both aerobic and hypoxic conditions (Table 3.3). A significant effect of hypoxia on gene expression was demonstrated by a shift in the ranking of endogenous gene stability when the hypoxic and aerobic samples were analysed together compared to the aerobic samples alone. *PGK1* was one of the top ranked control genes under aerobic conditions, but had the highest variability and was the least suitable control gene for both cell lines when hypoxic and aerobic samples were analysed together.

There was some disparity between the two cell lines, 22Rv1 and LNCaP, most notably for the genes *POLR2A* and *YWHAZ*. Analysis using Normfinder showed that, of the 16 HKGs analysed, *POLR2A* performed the best for the 22Rv1 samples, with the lowest variability at 0.2026. For LNCaP, however, it was ranked 15th, with a variability of 0.8485. Conversely, *YWHAZ* was the best control gene for the LNCaP samples (variability = 0.2238) but was 12th for 22Rv1 (variability = 0.7686).

Table 3.1: Genes tested for suitability as endogenous control genes for qRT-PCR

Symbol	Gene name	Function
18S rRNA	18s ribosomal RNA	Ribosome subunit
ACTB	β -actin	Cytoskeletal structural protein
B2M	β -2-microglobulin	β -chain of the major histocompatibility complex class I molecules
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis
GUSB	β -glucuronidase	Lysosome exoglycosidase
HMBS	Hydroxymethylbilane synthase	Heme biosynthesis
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Purine generation through the purine salvage pathway
IPO8	Importin 8	Nuclear protein import
PGK1	Phosphoglycerate kinase 1	Glycolysis enzyme
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	Synthesis of messenger RNA
PPIA	Cyclophilin A	Serine-threonine phosphatase inhibitor
RPLPO	ribosomal protein, large, P0	Ribosome subunit
TBP	TATA box binding protein	Initiation of transcription by RNA polymerase II
TFRC	Transferrin receptor (p90, CD71)	Cellular iron uptake
UBC	Ubiquitin C	Ubiquitination
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Signal transduction

Table 3.2: Mean Ct values for endogenous control genes

	22Rv1		LNCaP	
	21% oxygen	0.5% oxygen	21% oxygen	0.5% oxygen
<i>18S</i>	9.65 (0.53)	10.31 (2.42)	13.1 (2.98)	10.07 (0.3)
<i>ACTB</i>	19.53 (0.05)	20.82 (1.94)	23.44 (1.27)	22.39 (0.41)
<i>B2M</i>	24.05 (0.13)	25.59 (2.92)	26.33 (1.62)	25.61 (0.9)
<i>GAPDH</i>	19.56 (0.19)	19.18 (2.07)	23.7 (2.42)	20.53 (0.99)
<i>GUSB</i>	24.42 (0.12)	25.55 (1.48)	26.27 (0.59)	25.86 (0.6)
<i>HMBS</i>	26.03 (0.12)	27.17 (1.62)	28.48 (1.24)	27.81 (0.29)
<i>HPRT1</i>	24.67 (0.02)	26.44 (3.39)	30.43 (2.35)	29.19 (0.68)
<i>IPO8</i>	25.52 (0.04)	27.94 (3.99)	27.8 (0.68)	27.24 (0.38)
<i>PGK1</i>	23.78 (0.08)	21.78 (1.05)	25.55 (1.39)	21.68 (0.91)
<i>POLR2A</i>	24.45 (0.11)	25.34 (1.92)	26.3 (0.14)	26.53 (0.62)
<i>PPIA</i>	20.22 (0.09)	21.06 (1.38)	22.93 (1.55)	22.24 (0.4)
<i>RPLPO</i>	20.44 (0.14)	20.58 (1.03)	23.75 (2.79)	21.46 (0.59)
<i>TBP</i>	27.66 (0.03)	29.48 (3.03)	32.66 (3.72)	30.62 (0.75)
<i>TFRC</i>	23.99 (0.08)	25.93 (3.49)	26.85 (1.25)	25.81 (0.65)
<i>UBC</i>	22.54 (0.19)	23.13 (1.51)	26.27 (2.07)	24.56 (0.42)
<i>YWHAZ</i>	24.01 (0.16)	25.65 (3.56)	27.53 (1.4)	26.29 (0.72)

Mean Ct (standard deviation) of 3 independent biological replicates

The three endogenous control genes that performed best in 21% and 0.5% oxygen in both cell lines (*ACTB*, *PPIA* and *UBC*), were selected for further testing, along with the worst performer (*PGK1*) (Figure 3.1) in another prostate cancer cell line, DU145. For this analysis on four HKGs, Normfinder was unsuitable so the coefficient of variation ($C_tCV\%$) between the two treatment groups (21% and 0.5% oxygen) was calculated for the average C_t values of the three biological replicates within each group. Of the four genes, *PPIA* performed the best, with a $C_tCV\%$ of 0.53, whilst *PGK1* remained the poorest ($C_tCV\% = 7.44$) (Table 3.4).

Table 3.3: Endogenous control genes ranked by their stability in normoxic (21% oxygen) and hypoxic (0.5%) samples using Normfinder

22Rv1					LNCaP				
Normoxia			Normoxia & Hypoxia		Normoxia			Normoxia & Hypoxia	
Rank	Gene	Variability	Gene	Variability	Rank	Gene	Variability	Gene	Variability
1	ACTB	0.0136	POLR2A	0.2026	1	B2M	0.0366	YWHAZ	0.2238
2	TBP	0.0136	ACTB	0.2735	2	PPIA	0.0366	UBC	0.2725
3	PGK1	0.0335	HMBS	0.3812	3	YWHAZ	0.1839	TFRC	0.2924
4	HPRT1	0.0492	18S	0.4248	4	PGK1	0.2055	ACTB	0.3011
5	IPO8	0.0689	GUSB	0.4255	5	UBC	0.3019	HPRT1	0.3843
6	HMBS	0.0907	PPIA	0.4438	6	ACTB	0.3729	PPIA	0.4654
7	POLR2A	0.1036	UBC	0.5213	7	TFRC	0.3992	RPLPO	0.5158
8	GUSB	0.1193	B2M	0.5574	8	HMBS	0.4217	B2M	0.5238
9	TFRC	0.1202	TBP	0.6488	9	HPRT1	0.647	GUSB	0.5489
10	PPIA	0.1285	HPRT1	0.7182	10	GAPDH	0.7268	IPO8	0.6062
11	RPLPO	0.1418	GAPDH	0.7631	11	IPO8	1.0552	HMBS	0.6203
12	B2M	0.1601	YWHAZ	0.7686	12	RPLPO	1.1392	TBP	0.7034
13	UBC	0.1618	RPLPO	0.7973	13	GUSB	1.1622	GAPDH	0.8252
14	GAPDH	0.1675	TFRC	0.8274	14	18S	1.3449	18S	0.8473
15	YWHAZ	0.2074	IPO8	1.0709	15	POLR2A	1.9466	POLR2A	0.8485
16	18S	0.5269	PGK1	1.5052	16	TBP	2.1437	PGK1	0.9536

Values represent the mean of three independent biological replicates

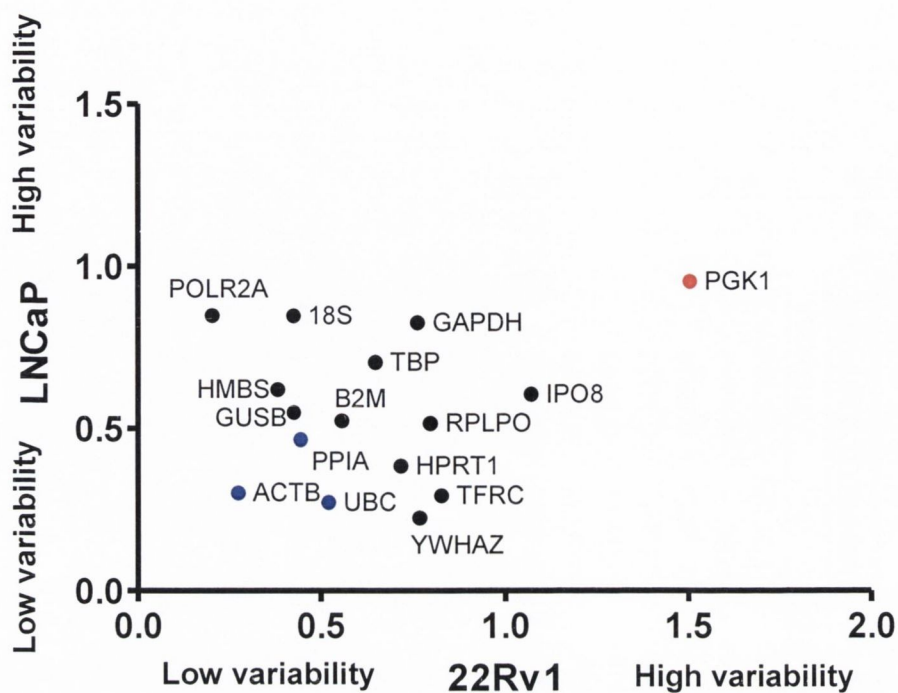


Figure 3.1: Variability of 16 endogenous control genes in prostate cancer cell lines 22Rv1 and LNCaP cultured in 21% or 0.5% oxygen. The three best performing genes (*ACTB*, *UBC*, *PPIA*, blue points), and the worst performing gene (*PGK1*, red point) are indicated and were selected for further analysis. Values represent mean of three independent replicates.

Table 3.4: Assessment of performance of endogenous control genes using coefficient of variation analysis in DU145 cells. Values represent the mean of three independent replicates.

	Average C _t Normoxia	Average C _t Hypoxia	Average C _t Normoxia & Hypoxia	SD	CtCV%
<i>ACTB</i>	18.88	20.11	19.5	0.86	4.43
<i>PPIA</i>	19.45	19.59	19.52	0.1	0.53
<i>UBC</i>	21.54	22.24	21.89	0.49	2.25
<i>PGK1</i>	21.67	19.51	20.59	1.53	7.44

3.2.2 Performance of selected endogenous control genes following longer exposure to 0.5% oxygen.

In the initial experiments, cells were cultured under aerobic (21% oxygen) or hypoxic (0.5%) conditions for 24 hours. Further analysis of the performance of the selected endogenous control genes was carried out on 22Rv1, LNCaP and DU145 cells grown in air or hypoxia for 48 hours before RNA extraction and assessment by C_t CV%. Longer exposure to 0.5% oxygen did not alter the ranking of the four endogenous controls by variability for any of the cell lines (Figure 3.2A).

The performance of the four selected endogenous control genes was tested in three additional cell lines: PC3, a metastatic prostate cancer cell line and the normal prostate lines PWR-1E and RWPE-1. The cells were grown in 21% or 0.5% oxygen for 48 hours prior to RNA extraction. The C_t CV% values for PC3 were not consistent with the previous results for 22Rv1, LNCaP and DU145, with *PGK1* appearing the most suitable endogenous control of the four genes tested (C_t CV% = 0.34). The best performing genes for PWR-1E and RWPE-1 were *UBC* (C_t CV% = 1.27) and *PPIA* (C_t CV% = 1.15) respectively (Figure 3.2B).

There was a large degree of variation between cell lines in the performance of the potential endogenous control genes. The most consistent genes were *UBC*, with the lowest C_t CV% across all six cell lines, ranging from 1.09 (22Rv1) to 3.52 (PC3) and *ACTB*, with C_t CV ranging from 0.33 (22Rv1) to 7.82 (PC3).

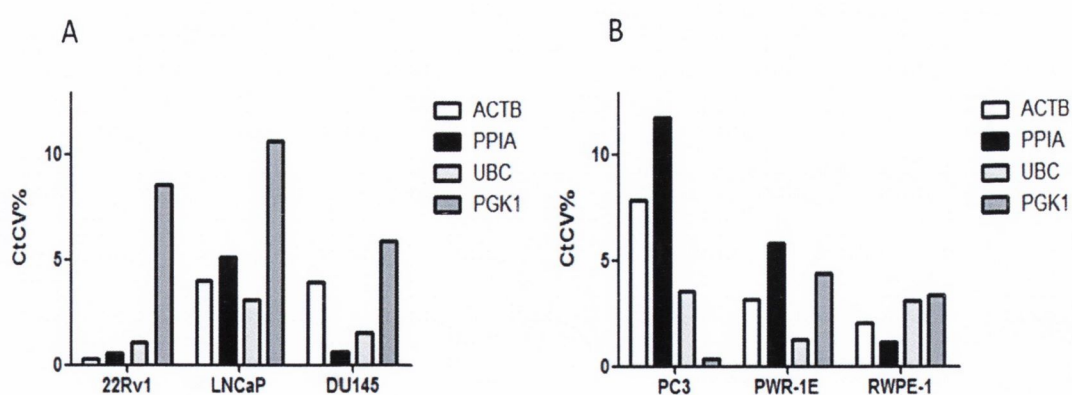


Figure 3.2: A) Coefficient of variation of C_t values between aerobic (21% oxygen) and hypoxic (0.5%) treatment groups for four candidate endogenous control genes in three prostate cancer cell lines following longer hypoxic exposure (48 hours). B)

Three additional prostate cell lines were tested after growth under aerobic or hypoxic conditions for 48 hours. $C_tCV\%$, coefficient of variation. Values represent mean of three independent replicates.

3.2.3 The choice of endogenous control can result in variation in gene expression results

The gene *HIF-1A* was selected for gene expression analysis. HIF-1 is regulated at the protein level and so gene expression levels remain stable between aerobic and hypoxia conditions. To demonstrate the importance of the endogenous control the two best performing genes (*ACTB* and *UBC*) were selected along with *PGK1*, whose expression was found to be altered by exposure to 0.5% oxygen in certain prostate cancer cell lines including 22Rv1. Each of these genes was tested as a control to normalise expression of *HIF-1A* in 22Rv1 (Figure 3.3). Both *ACTB* and *UBC* yielded similar levels of *HIF-1A* expression in aerobic and hypoxic cells, confirming constitutive expression of the gene. However, when *PGK1* was used to normalise results, an apparent 6-fold decrease in *HIF-1A* expression occurred under hypoxia ($p < 0.001$). This erroneous interpretation is directly due to the upregulation of *PGK1* under hypoxic conditions, leading to lower C_t values compared to the aerobic conditions (Table 3.2). Consequently, *HIF-1A* gene expression is falsely found to be downregulated under hypoxic conditions.

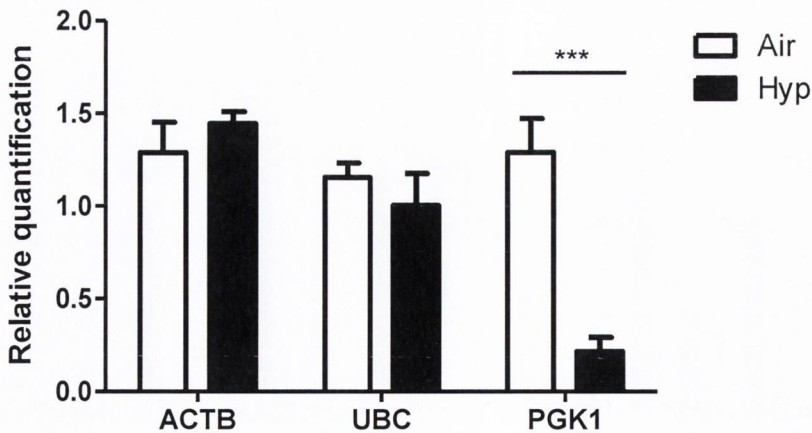


Figure 3.3: Relative quantification of *HIF-1A* mRNA. Values represent mean of three independent replicates $\pm$ standard error of the mean. ***, $p < 0.001$

3.2.4 *In silico analysis of hypoxia response elements*

Table A.1 in Appendix A shows the results of *in silico* analysis of hypoxia response elements in the endogenous control genes. This analysis was carried out by Dr. Stephen Madden of the National Institute for Cellular Biotechnology, Dublin City University, using a previously-published database containing information for 1,236 known or predicted transcription factor binding sites (TFBS) specific to HIF-1 α and HIF-1 β in the proximal promoter regions of around 20,000 genes.¹⁹⁸ The likelihood that the control genes contained HREs was assessed at four different position specific scoring matrix (PSSM) thresholds, 0.7, 0.75, 0.8, 0.85, each representing different degrees of sensitivity and selectivity. The higher the PSSM threshold the greater the similarity of the HRE to the previously experimentally characterised HREs used to generate the PSSM. At the highest threshold of 0.85, *PGK1*, *GAPDH* and *HMBS* were positive for a *HIF-1A* binding site in their proximal promoter. Both *PGK1* and *GAPDH* performed poorly as endogenous control genes for 22Rv1 and LNCaP. *HMBS* had high levels of variability for LNCaP cells but performed well for 22Rv1 as it was the third best of the 16 genes tested. The presence of HREs in their promoters suggests that these genes may be regulated in response to hypoxia, calling into question their use as endogenous controls under hypoxic conditions. However the results indicate that this *in silico* analysis alone is not sufficient to predict the variability of the potential endogenous control genes between aerobic and hypoxic samples.

3.2.5 *Performance of selected endogenous control genes in tumour and normal tissue*

The performance of the four HKGs was next tested in prostate tissues, by normalising the levels of *GSTP1* expression in 11 FFPE tumours relative to paired normal tissues. It has been widely established that *GSTP1* is downregulated in more than 90% of prostate tumours as a result of hypermethylation¹⁹⁹. Using *PPIA*, *UBC* or *PGK1* as endogenous controls showed that *GSTP1* levels were 2.5-3.9-fold lower in the tumour samples relative to the matched normal tissue (Figure 3.4). However, when *ACTB* was employed as the reference gene, *GSTP1* was found to be upregulated in 3 out of 11 tumours. The standard deviation for the average *GSTP1* expression level amongst the tumour samples is also large for *ACTB*. These results indicate its unsuitability as a housekeeping gene in FFPE tissues.



Figure 3.4: Average *GSTP1* mRNA expression levels in tumour samples relative to matched normal tissue using four different endogenous control genes. Values represent mean of 11 replicates $\pm$ standard deviation.

3.3 Discussion

This research highlights the importance of thorough investigation to find a suitable endogenous control gene before proceeding with qRT-PCR experiments. A growing number of research groups, including Bustin and colleagues, have demonstrated this and have drawn attention to the significance of the choice of endogenous control gene.²⁰⁰ Unfortunately, it is a consideration that is often overlooked.

What is clear from the research described in this chapter is that no universally suitable endogenous control gene exists; a gene shown to be suitable in one tissue type or cell line will not necessarily work for another. This research found some consensus between the cell lines but some obvious disparity too. As well as cell line to cell line variation, the treatment conditions can influence the expression of potential endogenous control genes. The possible effect that exposure to 0.5% oxygen could have on the expression of a panel of sixteen endogenous control genes has been addressed. Considering that hypoxia has been shown to have an effect on the expression of a wide-ranging number of genes, it was not surprising that when hypoxic samples were assessed with aerobic ones, there was a shift in the variability ranking for some of the candidate house-keepers. *In silico* analysis of the 16 potential endogenous control genes was carried out to assess the likelihood that they contain hypoxia response elements in their promoters. Using this technique, *PGK1* and *GAPDH* were both shown to be highly likely to have HREs, something which has been demonstrated previously.^{201, 202} This helps explain the high variability for these genes between samples maintained in 21% and 0.5% oxygen. However, *HMBS* and *POLR2A*, although highly likely to have HREs in their promoter regions, were two of the best performing endogenous control genes for 22Rv1 (but not for LNCaP). This *in silico* method of predicting the likelihood that genes contain HREs in their promoters was not sufficient to determine the performance of these genes as endogenous controls for qRT-PCR. These results underscore the complex nature of hypoxia-mediated gene regulation and the heterogeneity of its effect in different cell lines.

The considerable effect that the selection of endogenous control gene can have on the ultimate gene expression results was demonstrated through the use of a number of different genes to normalize data on *HIF-1A* gene expression levels in aerobic

and hypoxic 22Rv1 cell samples. It is known that HIF-1 α expression is controlled at the protein level and that transcription does not vary between normoxia and hypoxia.⁴¹ When either *ACTB* or *UBC* were used as the reference gene, levels of HIF-1 α gene expression were not significantly different between cell lines grown in air and hypoxia. As part of this study, these genes had been shown to be two of the best genes for use as endogenous controls. However when *PGKI* was used to normalize the data, *HIF-1A* was incorrectly found to be substantially downregulated in hypoxic samples, a result which is in fact due to the upregulation of *PGKI* under hypoxic conditions.

Gene expression can be modulated by the local tissue microenvironment leading to differences in expression levels between normal and tumour tissue from the same patient. Changes in the growth and metabolism characteristics of tumour cells can also mean differences in gene expression compared to normal tissue. The careful choice of endogenous control gene is just as crucial for qRT-PCR analysis of gene expression in tissue samples. It is also important to re-assess the suitability of endogenous control genes when moving from cell lines to tissue samples or *vice versa*. In this study *ACTB* was one of the best performing control genes for a number of prostate cancer cell lines. However, in patient tissue, it did not perform as well for some samples, with relatively high C_t values. The four candidate endogenous control genes *ACTB*, *PPIA*, *UBC* and *PGKI* were used to normalize results for expression of *GSTP1* in the tissue samples. *GSTP1* has been previously demonstrated to be downregulated in prostate tumour samples compared to normal tissue.¹⁹⁹ This finding was replicated here in all eleven tissue samples compared to the matched normal tissue when *PPIA*, *UBC* or *PGKI* were used as the endogenous control. However, when using *ACTB*, *GSTP1* was incorrectly found to be upregulated for three of the samples. These findings support those of Ohl *et al.* who found a significant difference in *ACTB* expression between matched malignant and benign prostate tissue.²⁰³ Erickson *et al.* suggested *ACTB* may be a good endogenous control gene for prostate cell line samples but not for tissue.²⁰⁴ *In vitro* growth conditions lead to steady high expression of *ACTB* in cell lines, whereas this high level of expression may not be present in tissue samples and this would affect the accuracy of results.

Mounting evidence highlights the importance of careful selection of the endogenous control genes in qRT-PCR experiments. However, such considerations are often overlooked. Failure to include this step means one cannot be confident in the ultimate gene expression findings, which may include erroneous and misleading results. This research has shown that the expression of some common endogenous control genes varies between aerobic and hypoxic samples, making them unsuitable for normalization of qRT-PCR results. Hypoxia is a common characteristic of solid tumours and a greater understanding of its effects on gene expression will improve our understanding of tumour biology. In this research, the hypoxia status of the patient tissue samples was not known. Future experiments could involve staining the tissue samples and correlating endogenous control performance with HIF-1 expression levels detected by immunohistochemistry to determine if the same variability in gene expression between aerobic and hypoxic cells is observed *in vivo*. qRT-PCR analysis described in subsequent chapters involved the cell lines 22Rv1 and LNCaP and *ACTB* was selected as the best performing control gene for these two cell lines when samples treated with 21% and 0.5% oxygen were being analysed together. Any future study involving qRT-PCR must use endogenous control genes that have been carefully validated.

Chapter 4

Characterisation of pPPE-CD activity
under hypoxic conditions

Chapter 4 Characterisation of pPPE-CD activity under hypoxic conditions

4.1 Introduction

4.1.1 pPPE-CD2.15 and pCMV-CD1.10 vectors

As discussed in chapter one (section 1.10) tissue or tumour targeting of GDEPT may help reduce the toxic side effects that can occur when a constitutively active promoter is used to control the expression of the enzyme. Despite direct transperineal injection of the vector, dissemination to other nearby organs, mainly the bladder and vas deferens, still occurs.¹⁶⁵ If organ-specific promoters were used, these tissues should not be damaged, as cell kill would be restricted to the prostate. The PSA promoter/enhancer has potential in this regard as PSA is produced specifically by the epithelial cells of the prostate.¹⁶⁸

Previous research in the Prostate Molecular Oncology Group by Dr. Ruth Foley had involved the construction of the vector pPPE-CD2.15 (referred to as pPPE-CD during this thesis), in which 0.7kb of the PSA promoter region and 1.6kb of the upstream enhancer region were cloned into pAMdPE1.1 (the eukaryotic expression vector pALTER-MAX with the CMV promoter and enhancer removed) upstream of the open reading frame (ORF) of the cytosine deaminase gene. As a positive control, the CD ORF was cloned downstream of the constitutively active CMV promoter and enhancer in the pALTER-MAX vector to make pCMV-CD1.10 (referred to as pCMV-CD).

CD expression in pPPE-CD transfected 22Rv1 and LNCaP cells was shown to be induced by DHT treatment. CD expression in PC3 cells, a PSA-negative cell line, was restricted to those transfected with pCMV-CD and did not occur in those transfected with pPPE-CD. This chapter describes further characterisation of pPPE-CD in light of strong evidence for an interaction between the androgen receptor and HIF-1 α signalling in prostate cancer cells. It was postulated that this 'cross-talk' may result in higher levels of activation of pPPE-CD under hypoxic (0.5% oxygen) conditions.

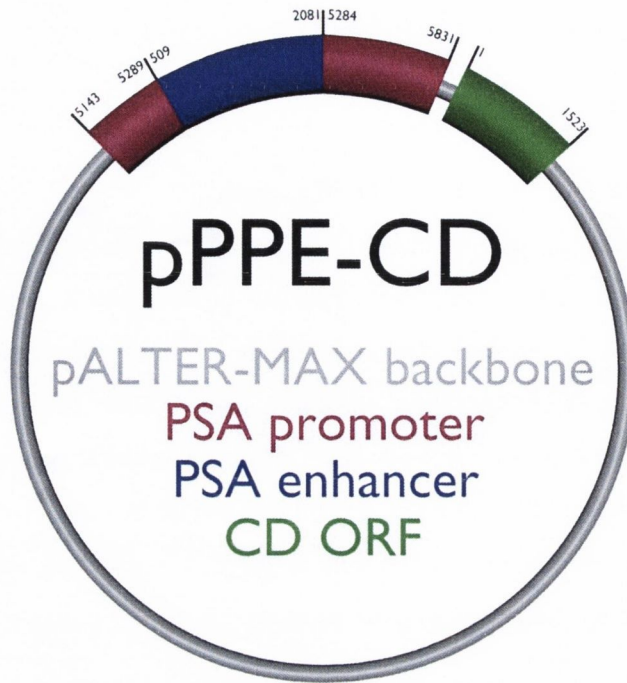


Figure 4.1: pPPE-CD vector showing location of PSA promoter and enhancer and CD open reading frame. Numbers represent the positions in the GenBank sequence.

4.1.2 AR/HIF-1 cross-talk

Mabjeesh *et al.* investigated the activation of vascular endothelial growth factor (VEGF) expression in prostate tumour cells.²⁰⁵ The expansion and metastasis of a tumour requires an adequate supply of blood. The growth of blood vessels from those already present (angiogenesis) is controlled by a host of factors, including VEGF.²⁰⁵ It was known that the transcription factor HIF-1 regulates VEGF and that androgen stimulation can induce VEGF expression.^{206, 207} However, the exact mechanisms that control this had not been elucidated. Through the use of the androgen DHT, as well as the anti-androgen flutamide, Mabjeesh *et al.* demonstrated that androgens upregulate HIF-1 α protein expression and VEGF production in LNCaP cells but not in PC3 cells, which do not express the androgen receptor.

This effect on HIF-1 α was shown to be translation-dependent. PC3 cells were grown in LNCaP conditioned medium. When LNCaP had been treated with DHT, a dose-dependent increase in HIF-1 α levels was observed in the PC3 cells.

Conversely, medium from flutamide treated cells reduced HIF-1 α levels. The results suggested the presence of secreted factors in the medium which affect HIF-1 α expression. Its expression had previously been shown to be controlled by epidermal growth factor (EGF), as part of the phosphatidylinositol-3'-kinase (PI3K)/AKT pathway.²⁰⁸ Conditioned medium from LNCaP treated with an antibody to EGF reduced HIF-1 α protein expression in PC3 cells. Use of LY294002, an inhibitor of tyrosine kinase receptor/PI3K, prevented HIF-1 α expression in LNCaP. The results led the group to put forward a hypothesis to explain the expression of VEGF in response to androgen stimulation: activation of AR results in its binding to the androgen response elements of various genes, leading to proliferation of the cells and secretion of growth factors including EGF. EGF can then bind to its receptor, activating the PI3K/AKT pathway and leading to HIF-1 α expression and the subsequent expression of VEGF and other target genes (Figure 4.2).

Following this publication, Boddy *et al.* used prostate cancer specimens obtained by radical prostatectomy to further study the relationship between AR, HIF-1 α and VEGF.²⁰⁹ There was a significant association between HIF-1 α and HIF-2 α expression and that of AR. In addition, VEGF was significantly associated with the AR and HIF-1 α and HIF-2 α . These *in vivo* results support the previous findings by Mabeesh *et al.*²⁰⁵

Rudolfsson *et al.* used a marker of hypoxia, pimonidazole, to observe changes in prostate hypoxia levels in castrated rats.²¹⁰ Testosterone treatment 7 days after castration resulted in increased hypoxia in the epithelium. HIF-1 α , VEGF and carbonic anhydrase 9 (another HIF-1 target gene) mRNA and protein levels were also increased by testosterone treatment. Experiments involving the normal prostate epithelial cell line RWPE-1 and Gefitinib, an inhibitor of the EGF receptor, demonstrated that androgens regulate HIF-1 α in non-malignant cells in the same manner postulated by Mabeesh *et al.*, as the inhibitor caused a reduction in HIF-1 α levels when given alongside EGF or R1881, a synthetic androgen. *In vivo* however, Gefitinib did not reduce HIF-1 α levels in castrated testosterone-stimulated rats but did reduce VEGF expression. The group concluded that there may in fact be two ways in which androgens can stimulate HIF-1 α , through the PI3K/AKT pathway but also through a transient rise in hypoxia due to high levels of metabolism but

insufficient blood flow. The increase in VEGF that results may contribute to the growth of the prostate in response to androgens.

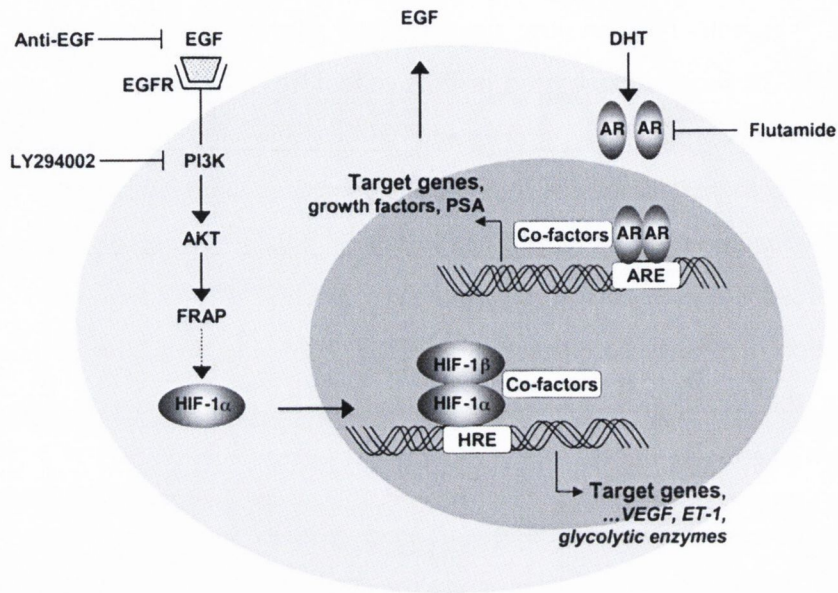


Figure 4.2: Mechanism proposed by Mabjeesh *et al.* for HIF-1α upregulation in LNCaP cells in response to DHT stimulation.²⁰⁵

Park *et al.* used the androgen-responsive cell line, LNCaP, to examine the effect of hypoxia on the behaviour of AR.²¹¹ Cells were placed in a hypoxic environment for 4 hours, then analysed immediately or reoxygenated for 2 - 24 hours. Binding of the AR to androgen response elements (AREs) was observed by electromobility gel shift assay to increase significantly after transient hypoxic treatment and was highest after 2 hours of reoxygenation. Because reoxygenation led to a decrease in HIF-1α binding activity and protein levels, Park *et al.* proposed that HIF-1α signalling was not responsible for the upregulation of AR activity previously observed.

The functional significance of the change in AR activity following hypoxia and reoxygenation was tested using PSA, with mRNA and protein levels found to be higher than in untreated cells. This effect extended to an ARE-luciferase construct transfected into LNCaP cells, with a significant increase in luciferase activity for 0, 2 and 6 hours of reoxygenation after hypoxia. Park *et al.* demonstrated that transient

hypoxia affected the subcellular location of AR, stimulating its movement from the cytosol into the nucleus, as well as increasing its presence at the AREs of the PSA gene. Growing LNCaP cells in charcoal stripped FBS prior to hypoxia exposure abolished AR binding activity. This could be restored using the synthetic androgen R1881, suggesting the effect of hypoxia is dependent on the presence of androgen. When CD-FBS medium was supplemented with increasing concentrations of R1881 in air or transient hypoxia, ARE-luc activity was increased in hypoxia compared to the aerobic control for each concentration, especially at lower levels of R1881.

The relationship between hypoxia and AR was also investigated by Horii *et al.*¹⁹⁰ DHT was shown to enhance hypoxia-stimulated expression of GLUT-1, a gene containing hypoxia response elements (HREs) in its promoter. Using a HRE-containing reporter gene, pHRE-Luc, DHT was again shown to enhance hypoxia stimulated transcription in LNCaP cells and in COS-7 cells transfected with AR. The presence of DHT increased HIF-1 α protein levels also.

Synergistically, the levels of PSA mRNA increased when hypoxia was combined with DHT stimulation. After further investigation, Horii *et al.* suggested this effect may be PSA specific as hypoxia increases expression of pPSA4.5-Luc, which contains the 5'-flanking region of the PSA gene, but not pARE-Luc, which contains two copies of the ARE. Also, hypoxia did not enhance expression of *NKX3.1* or *PMEPA1*, other AR regulated genes. This was in contrast to some of the work of Park *et al.*, who showed that hypoxia alone was sufficient to increase luciferase expression in pARE-Luc transfected LNCaP.²¹¹ To elucidate which part of the PSA promoter is involved, pPSA4.5-Luc deletion mutants were created by Horii *et al.* and it was found that the area from -3963 to -3932 is crucial for the upregulation of expression by hypoxia. Through site specific mutation, they identified the sequence 5'-RCGTG-3' that acts as a HRE and is crucial for HIF-1 binding to the PSA promoter.

Horii *et al.* showed that the cross talk between the AR and HIF-1 α is a result of a physical interaction between the two. This was demonstrated using co-immunoprecipitation and LNCaP cells grown under hypoxia & androgen positive conditions. One of the AREs (ARE III) of the PSA promoter is located near the HRE. Chromatin immunoprecipitation from DHT and hypoxia-treated cells with an

anti-AR antibody, followed by PCR of the immunoprecipitates showed that both ARE III and HRE were linked with the AR. This shows HIF-1 interacts with the AR at this region in the promoter. The levels of ARE III were higher when cells were treated with hypoxia and DHT compared to DHT alone. Horii *et al.* hypothesised that HIF-1 may stabilise the AR transcriptional complex, thereby acting to upregulate PSA expression, known to be involved in prostate cancer growth and metastasis.

Mitani *et al.* have also investigated the relationship between hypoxia and AR regulation of gene expression.²¹² They looked at the response of LNCaP cells, transiently transfected with pARE-Luc, followed by exposure to a range of DHT concentrations in hypoxia (1% O₂) or normoxia (21%) for nine hours. They observed an enhancement of AR transactivation under hypoxic conditions but only when combined with low levels of DHT (0.05 and 0.1 nM DHT). The enhancement of DHT (0.1 nM) activation of pARE-Luc by hypoxia was repressed by siRNA knockdown of HIF-1 α . This could be reversed when HIF-1 α (mut), an siRNA resistant and oxygen independent form of HIF-1 α , was overexpressed in the siRNA-treated cell line.

The expression levels of two androgen-responsive genes, PSA and NKX3.1, were measured in air and hypoxia, with and without DHT (0.1nM). Levels of PSA were enhanced in hypoxia both in the presence and absence of DHT addition. In the case of NKX3.1, hypoxia increased expression only when DHT was also present. HIF-1 α siRNA inhibited the increase in NKX3.1 mRNA in hypoxia, indicating it has role in this process.

The group went on to show that the HIF-1 binding to HREs is not necessary for the AR transactivation in hypoxia at 0.1 nM DHT. They used mutated versions of HIF-1 α , HIF-1 α (A26E) and HIF-1 α (R30A), which can not bind to HREs and are not repressed by siRNA for HIF-1 α . When HIF-1 α (A26E) and HIF-1 α (R30A) were expressed in HIF-1 α siRNA treated LNCaP cells, the inhibition of HIF-1 activity remained but the hypoxia induced upregulation of androgen-induced gene expression of pARE-Luc still occurred. siRNA knockdown of HIF-1 β /ARNT, with which HIF-1 α forms a heterodimer to form HIF-1 which then binds to HREs, had no effect on this transactivation at 0.1nM DHT.

These results are in contrast to some of the findings of Horii *et al.* who did not observe an enhancement in transcription of pARE-Luc or the gene NKX3.1 when hypoxia treatment was combined with DHT and who suggested the upregulation of DHT stimulated expression by hypoxia was restricted to the PSA gene.¹⁹⁰ However, Mitani *et al.* had used a range of DHT concentrations (0.05 – 10 nM) to test pARE-Luc and it was only at low concentrations of DHT (0.05 and 0.1 nM DHT) that there was a significant difference between aerobic and hypoxic samples. Horii *et al.* were using higher levels of DHT, 1 nM and 10 nM for pARE-Luc and 10 nM for NKX3.1.

It is now clear that the combination of hypoxia with androgen stimulation results in a synergistic upregulation of expression of certain genes previously known to be either hypoxia or AR-regulated. Research has begun to explain the mechanisms that control this cross-talk, despite some differences in the findings of the various research groups. Further study of this phenomenon would help our understanding of prostate cancer progression and treatment response.

4.1.3 Aims and objectives

Emerging evidence suggests that HIF-1 interacts with AR in driving the PSA promoter. This research aimed to investigate this AR/HIF-1 cross-talk to determine the potential of a GDEPT approach that may allow transcriptional control of the vector pPPE-CD by hypoxia, as well as androgen.

Aim: To determine if this cross-talk plays a role in the activation of pPPE-CD; and if so, if it is mediated by the same HIF-1 interaction.

Aim: To assess whether the cross-talk leads to increased efficacy of 5-FC treatment in pPPE-CD-transfected cells.

4.2 Results

4.2.1 *Determination of optimal concentration of DHT for PSA expression*

To determine the optimal concentration of DHT for PSA upregulation, LNCaP and 22Rv1 grown in media containing 1% pen/strep and 10% charcoal dextran stripped FBS (CD-FBS) for 72 hours, followed by treatment with a range of DHT concentrations under aerobic (21% oxygen) or hypoxic (0.5%) conditions for a further 72 hours. Subsequent assessment of the PSA levels secreted by the cells in to the surrounding media was carried out using an EIA and the levels of protein detected were normalised to cell number using an MTT assay. For LNCaP cells, PSA levels showed a bell-shaped dose response with increased expression with rising DHT concentration up to 1000 nM, above which PSA protein levels decreased (Figure 4.3 A). There was a highly statistically significant increase in PSA protein levels for 100, 1000 and 5000 nM DHT ($p < 0.001$, one-way ANOVA with Bonferroni post-hoc testing) compared to 0 nM. The difference was less but still significant for 10,000 nM DHT ($p < 0.05$). The PSA levels for 22Rv1 were much lower than LNCaP. DHT stimulation resulted in a significant increase in PSA protein expression ($p < 0.001$ for 10 nM, 1000 nM, 5000 nM, 10,000 nM, $p < 0.05$ for 100 nM) (Figure 4.3 B). 1000 nM of DHT was selected as the optimal concentration for PSA expression for both cell lines. At this concentration LNCaP produces approximately 20-fold higher levels of PSA than 22Rv1.

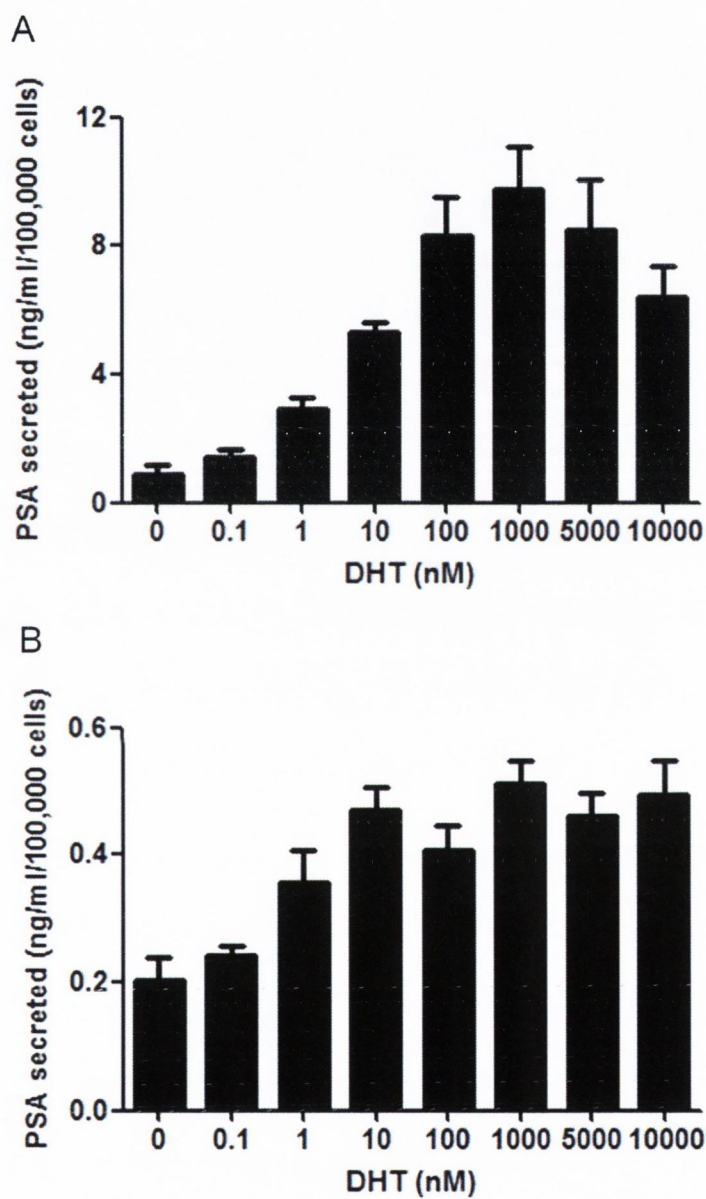


Figure 4.3: PSA expression following 72 hours of DHT treatment in LNCaP (A) and 22Rv1 (B) Values represent the mean of three independent replicates $\pm$ standard error of the mean

4.2.2 Investigation of AR/HIF-1 cross-talk and its effect on the activation of pPPE-CD

PSA expression in response to hypoxia

Previous research has suggested that there is cross-talk between AR and HIF-1 in the control of gene expression and that *PSA* expression levels are controlled by this synergistic relationship, with higher levels of expression in LNCaP cells when DHT treatment is combined with hypoxia.¹⁹⁰ To confirm that this was the case under the experimental conditions being used here, LNCaP cells were treated with a range of DHT concentrations and grown for 72 hours in either 21% or 0.5% oxygen. A PSA ELISA was carried out on the amount of secreted PSA protein in the media and this was normalised to cell number using an MTT assay. For LNCaP cells the levels of secreted PSA were significantly higher for the hypoxic cells when the three higher concentrations of DHT (10, 100 and 1000 nM) were used (Figure 4.4). The experiment was repeated for the 22Rv1 cell line. In this case there was no significant difference between the aerobic and hypoxic samples for any of the DHT concentrations (Figure 4.5).

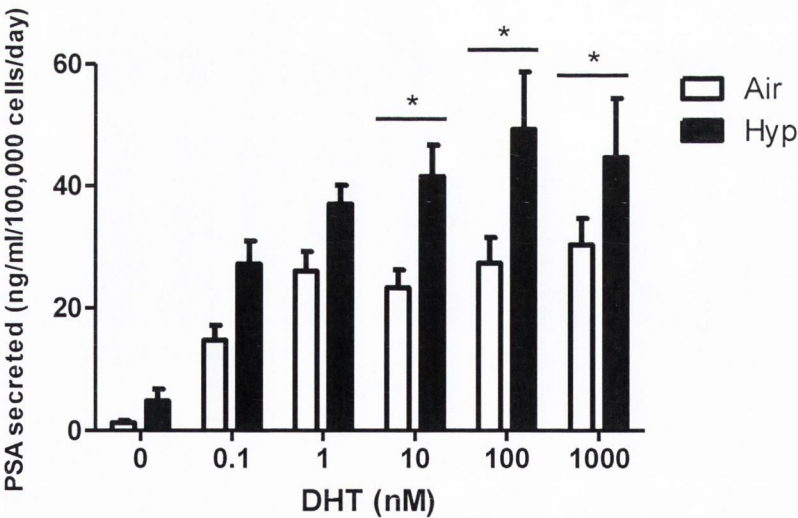


Figure 4.4: PSA protein levels measured by PSA ELISA following DHT stimulation in LNCaP under aerobic and hypoxic conditions. Hyp, hypoxia (0.5% oxygen); values represent mean of three independent replicates $\pm$ standard error of the mean; *, $p < 0.05$.

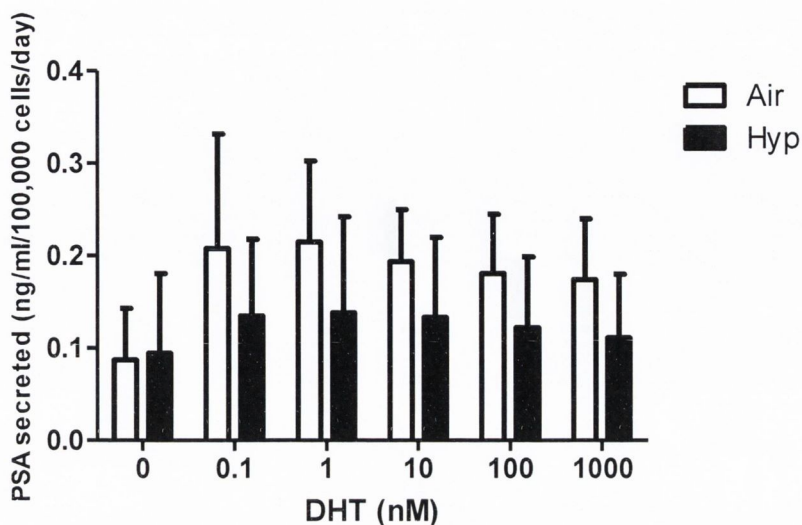


Figure 4.5: PSA protein levels measured by PSA ELISA following DHT stimulation in 22Rv1 under aerobic and hypoxic conditions. Hyp, hypoxia (0.5% oxygen); values represent mean of three independent replicates $\pm$ standard error of the mean.

Further investigation of PSA expression in response to DHT and hypoxia treatment was carried out using qRT-PCR. These results agreed with the previous findings of the PSA EIAs: in LNCaP cells, growth in 0.5% oxygen resulted in a significant increase in PSA expression compared to aerobic samples (21% oxygen) ($p < 0.01$ for 0.1 nM DHT, $p < 0.001$ for 1 nM and $p < 0.05$ for 10 nM, Two-way ANOVA with Bonferroi post-hoc testing, Figure 4.6). For 22Rv1 there was no significant difference between PSA expression levels under aerobic and hypoxic conditions (Figure 4.7).

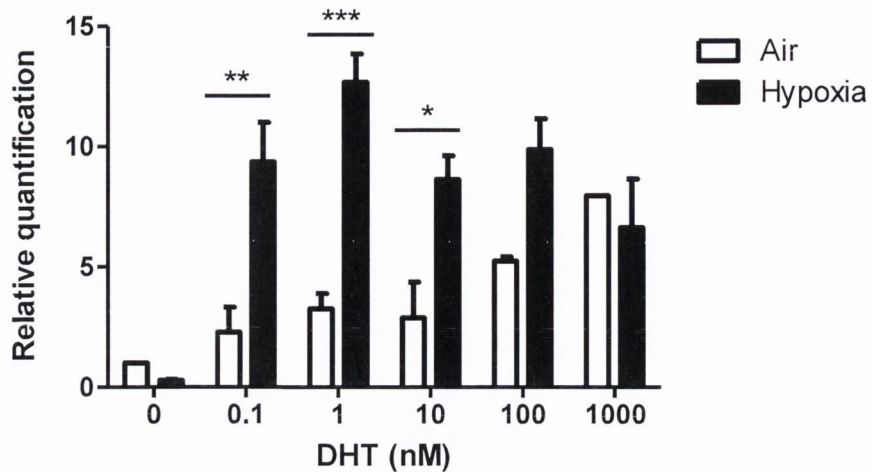


Figure 4.6: Relative quantification of *PSA* mRNA in aerobic (21% oxygen) and hypoxic (0.5%) LNCaP samples treated with increasing concentrations of DHT. Values represent mean of three independent replicates $\pm$ standard error of the mean.

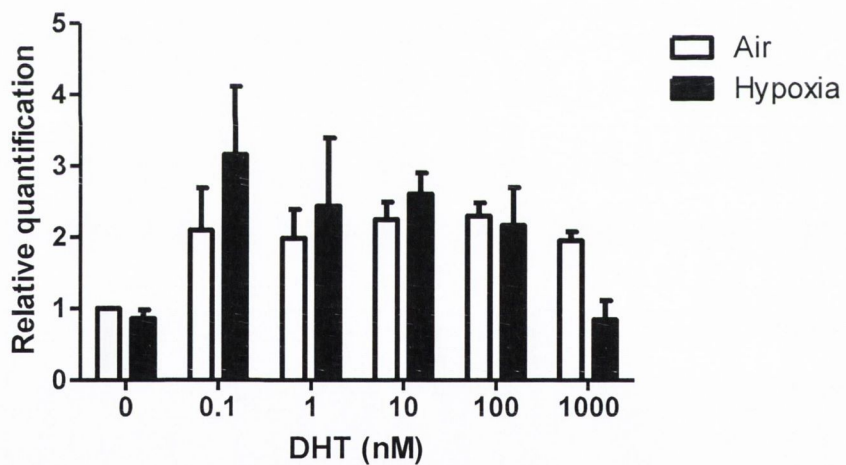


Figure 4.7: Relative quantification of *PSA* mRNA in aerobic (21% oxygen) and hypoxic (0.5%) 22Rv1 samples treated with increasing concentrations of DHT. Values represent mean of three independent replicates $\pm$ standard error of the mean.

pPPE-CD expression in response to hypoxia

To assess whether the apparent cross-talk between AR and HIF-1 gene regulation would affect activation of pPPE-CD, where CD is downstream of the PSA promoter, LNCaP cells were transfected with pPPE-CD and pCMV-CD (positive control) with untransfected cells as a negative control. The cells were treated with 1000 nM DHT or media alone in air (21% oxygen) or hypoxia (0.5%) for 72 hours. Protein was extracted from the cells and Western blotting for CD was carried out. In LNCaP cells hypoxia alone was not sufficient to activate CD expression. DHT stimulation resulted in detectable CD levels, which were increased when DHT treatment was combined with growth in a hypoxic environment (

Figure 4.8). Actin was used as a loading control.

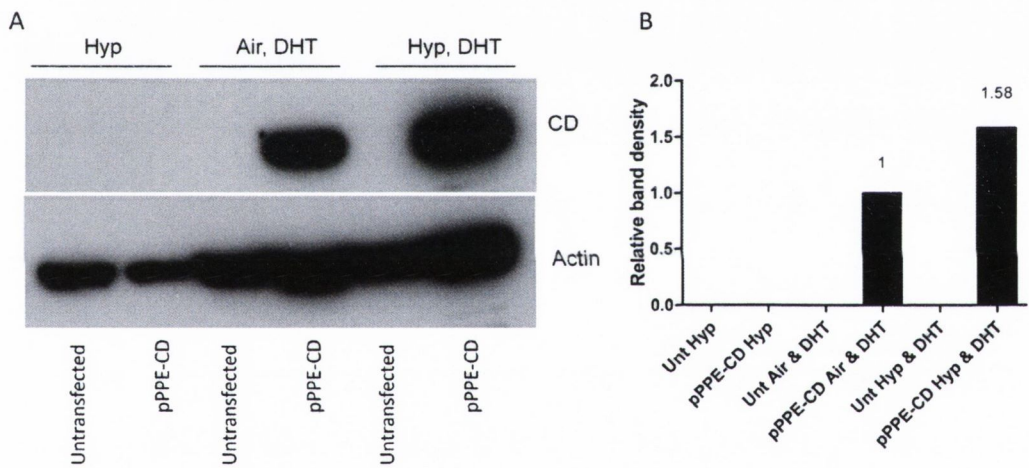


Figure 4.8: A) Western blotting for cystosine deaminase protein expression in pPPE-CD transfected LNCaP cells in response to various treatments. Actin levels were used as a loading control. Representative blot, N=2. Hyp, hypoxia; DHT, 1000nM DHT; Unt, untransfected. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

The experiment was also carried out with 22Rv1 cells. In pPPE-CD transfected 22Rv1 cells, the expression of CD was upregulated when hypoxia was combined with androgen stimulation in comparison to DHT alone. In this cell line, hypoxia alone was sufficient to activate the pPPE-CD vector (Figure 4.9).

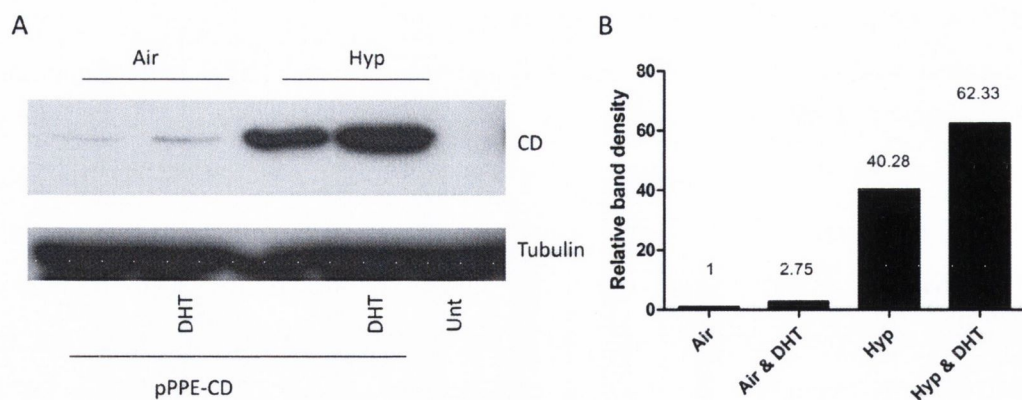


Figure 4.9: A) Cytosine Deaminase protein expression in pPPE-CD transfected 22Rv1 cells in response to various treatments. Tubulin levels were used as a loading control. Representative blot, N=2. Hyp, hypoxia; DHT, 1000nM DHT. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

The results of these Western blots suggest that the synergistic relationship between the AR and HIF-1 results in higher CD expression in pPPE-CD transfected cells when growth in 0.5% oxygen and DHT treatment are combined. To further test this theory, cobalt chloride, a chemical known to stabilise HIF-1 α , was used as a mimic of hypoxia. Confirmation of the stabilisation of HIF-1 α following cobalt chloride treatment was obtained using Western blotting (Figure 4.10). Untransfected cells and cells transiently transfected with pPPE-CD were treated with DHT (1000 nM), cobalt chloride (1 μ l/ml) and the two combined. Western blotting for CD on LNCaP/pPPE-CD and 22Rv1/pPPE-CD cells treated with cobalt chloride shows that HIF-1 α stabilisation replicates the effect previously observed under hypoxic conditions, with an upregulation of CD expression for cobalt chloride treatment alone and in combination with DHT (Figure 4.11 and Figure 4.12).

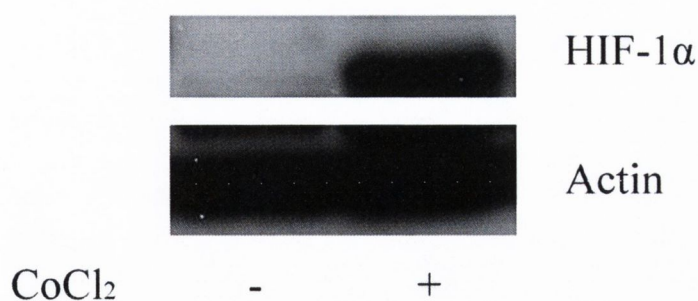


Figure 4.10: HIF-1 α protein expression in 22Rv1 cells treated with cobalt chloride. CoCl₂, cobalt chloride.

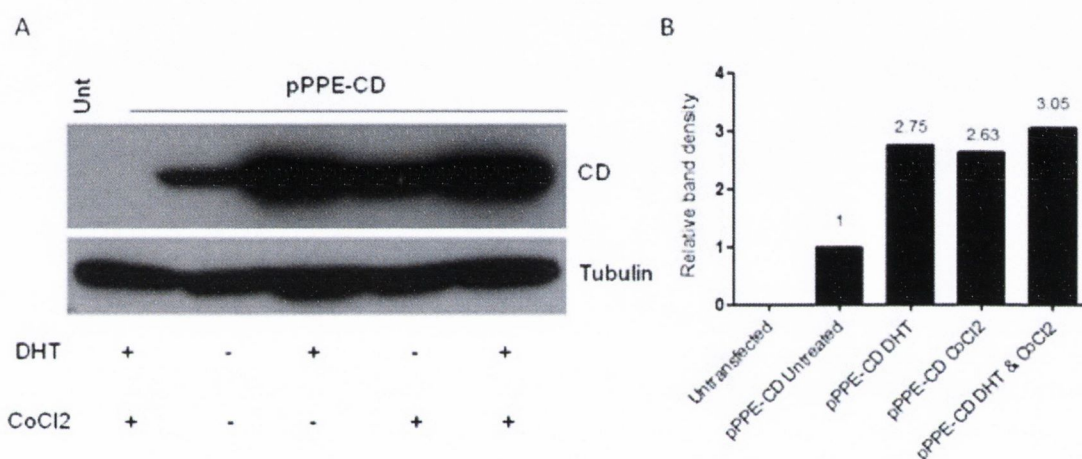


Figure 4.11: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected LNCaP cells treated with cobalt chloride and DHT. Tubulin levels were used as a loading control. Representative blot, N=2. CoCl₂, cobalt chloride; DHT, 1000nM DHT. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

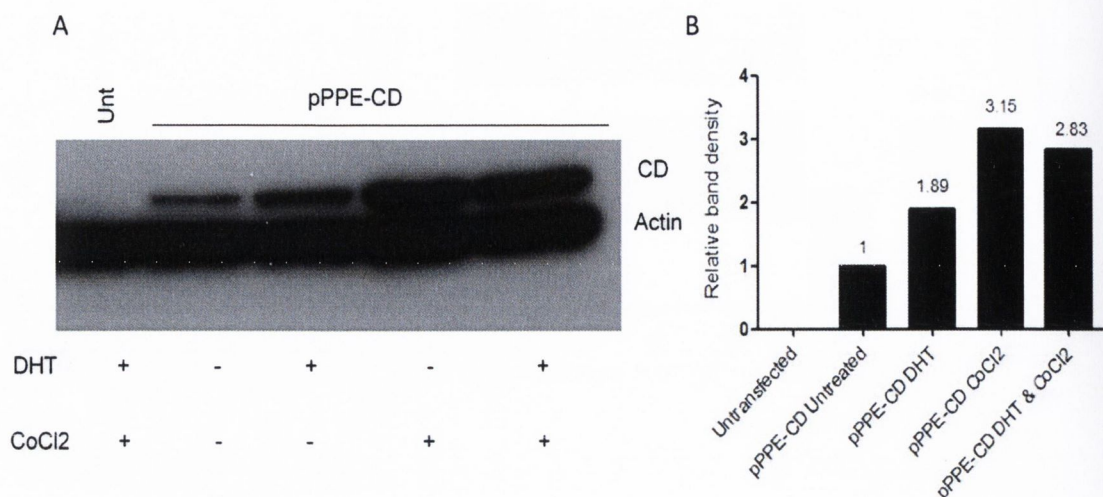


Figure 4.12: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected 22Rv1 cells treated with cobalt chloride and DHT. Tubulin levels were used as a loading control. Representative blot, N=2. CoCl₂, cobalt chloride; DHT, 1000nM DHT. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

Inhibition of HIF-1A

To further examine the role of HIF-1 in the regulation of CD expression in pPPE-CD transfected cells, attempts were made to knock-down *HIF-1A* expression using siRNA. Despite successful knockdown with 24 hour siRNA treatment in 22Rv1 cells (relative quantity reduced to 0.0182 compared to control) and to a lesser extent LNCaP (relative quantity 0.57) (Figure 4.13) this could not be consistently replicated in subsequent experiments. The well to well variation in levels of *HIF-1A* knockdown suggested further substantial optimization or alternative siRNAs/transfection systems would be needed before this could be used to test CD expression in pPPE-CD transfected cells grown in 0.5% oxygen in the absence of HIF-1A.

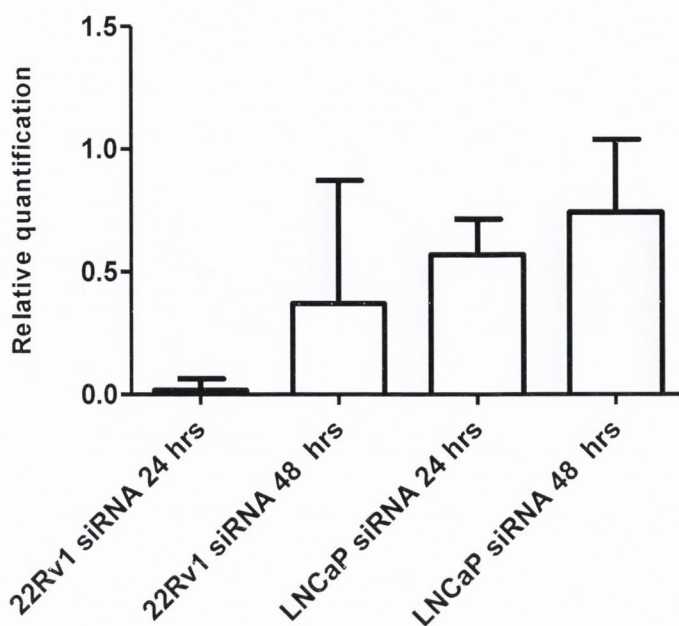


Figure 4.13: Relative quantification of *HIF-1A* mRNA levels in siRNA transfected samples compared to their matched untreated controls.

Following the attempted siRNA knockdown, an inhibitor of HIF-1 α was tested, 3-(2-(4-Adamantan-1-yl-phenoxy)-acetylamino)-4-hydroxybenzoic acid methyl ester (Merck). The inhibitor works by preventing the cellular accumulation of HIF-1 α under hypoxic conditions (without affecting levels of *HIF-1A* mRNA or HIF-1 β protein), thereby preventing HIF-1 transcriptional activity.

Protein samples were collected from cells grown in 21% oxygen alone and in 21% oxygen with cobalt chloride for use as a negative and positive control for HIF-1 α expression respectively. Untreated cells grown in hypoxia (0.5% oxygen) were also used as a positive control. The powdered drug was dissolved in DMSO and increasing concentrations were added to the 22Rv1 cells. 16 hours later, protein was extracted for Western blotting. Merck reported that the IC₅₀ was 0.7 μ M in AGS and 2.6 μ M in Hep3B cells.²¹³ Coutts *et al.* have successfully used it to inhibit HIF-1 α in MCF-7 cells at a concentration of 30 μ M.²¹⁴ In this experiment, initial treatments of 22Rv1 cells with 1 to 10 μ M did not decrease HIF-1 α levels (Figure 4.14). The dose was increased but a dose as high as 100 μ M had no effect on HIF-

1 α protein expression in 22Rv1 cells and the inhibitor was not used in any further experiments (Figure 4.15).

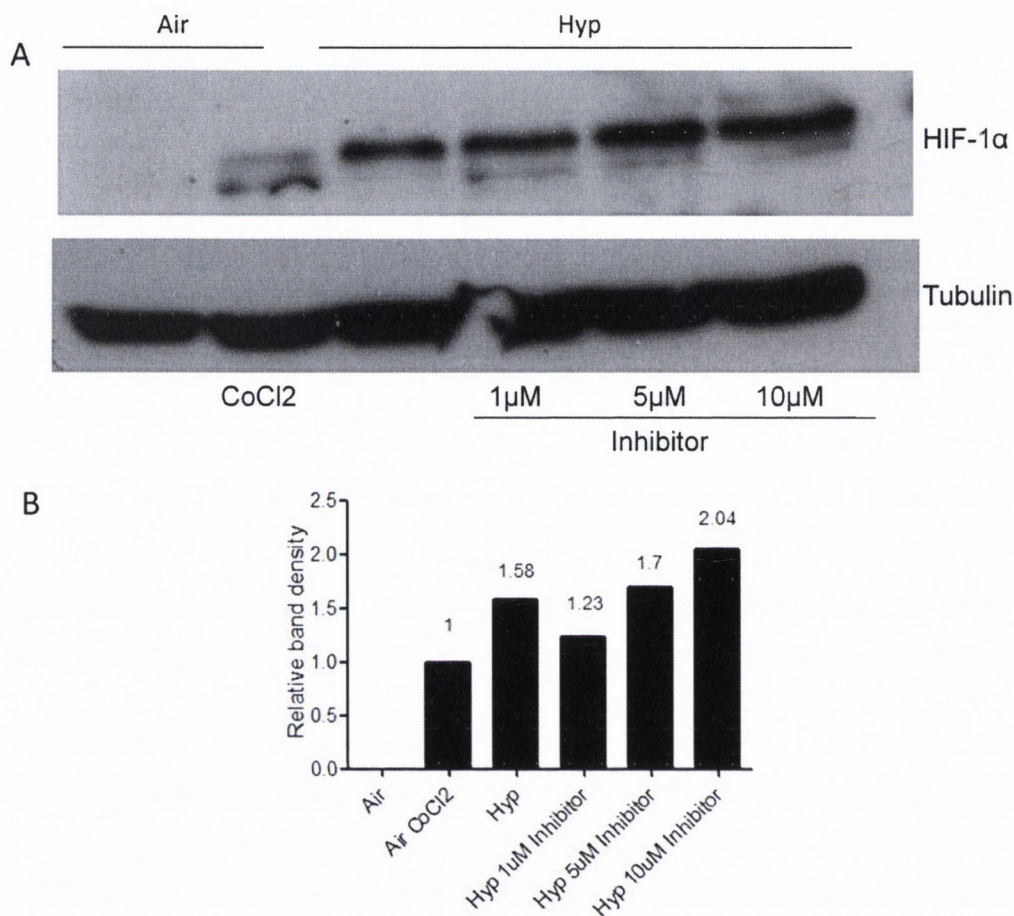


Figure 4.14: A) Western blotting for HIF-1 α protein expression in 22Rv1 cells treated with increasing concentrations of the HIF-1 α inhibitor under hypoxic conditions. Tubulin was used as a loading control. Hyp, hypoxia; CoCl₂, cobalt chloride; inhibitor, 3-(2-(4-Adamantan-1-yl-phenoxy)-acetyl-amino)-4-hydroxybenzoic acid methyl ester. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

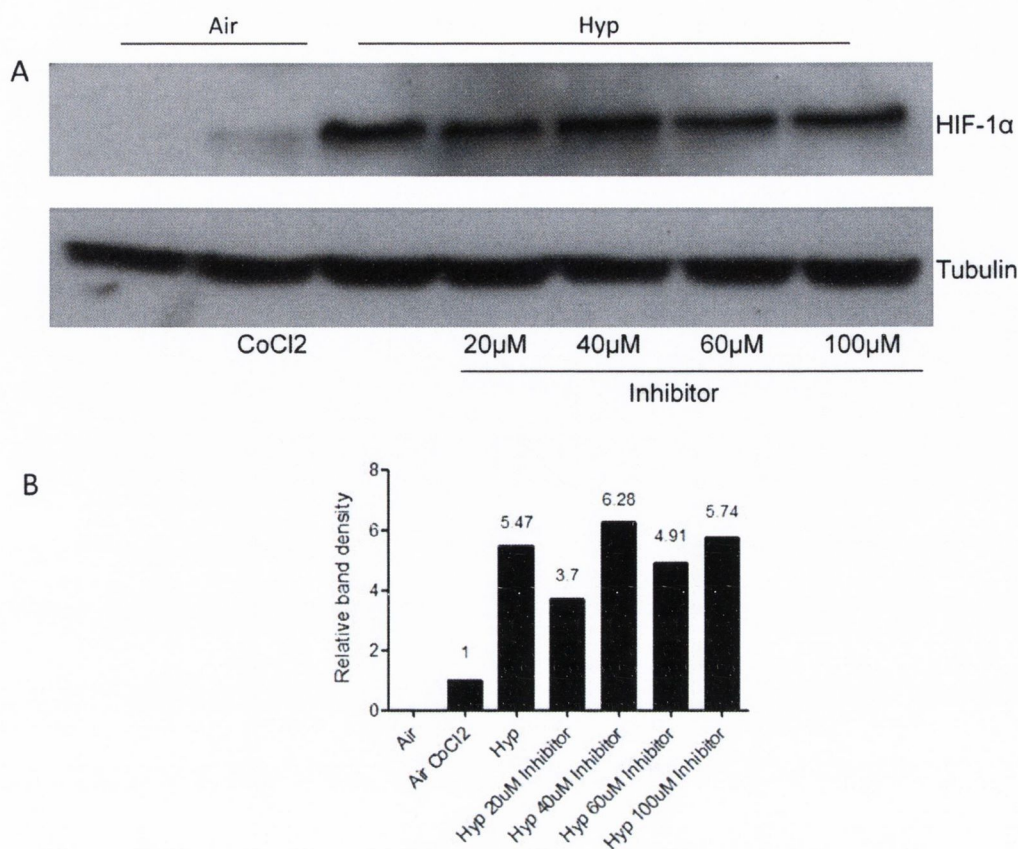


Figure 4.15: A) Western blotting for HIF-1α protein expression in 22Rv1 cells treated with increasing concentrations of the HIF-1α inhibitor under hypoxic conditions. Tubulin was used as a loading control. Hyp, hypoxia; CoCl₂, cobalt chloride; inhibitor, 3-(2-(4-Adamantan-1-yl-phenoxy)-acetylamino)-4-hydroxybenzoic acid methyl ester. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

Cell viability following pPPE-CD/5-FC treatment under aerobic and hypoxic conditions

The results have indicated that the activation of CD expression in pPPE-CD transfected cells can be increased by growing the cells in a hypoxic environment. The next step was to assess if this would affect the levels of cell kill when the transfected cells were treated with various concentrations of 5-FC. 5-FC is converted to its toxic form 5-FU by CD, so an increase in CD levels should increase the level of toxicity.

Cell viability assays (MTT assays) were carried out to examine the effect of various treatment conditions on the number of pPPE-CD transfected LNCaP and 22Rv1 cells. Untransfected cells were used as a negative control and pCMV-CD transfected cells were used as a positive control. Following transfection, an MTT assay was carried out on sample wells for each set of cells (untransfected, pPPE-CD-transfected, pCMV-CD-transfected). Following growth of the cells with or without DHT in aerobic (21% oxygen) and hypoxic conditions (0.5%) and 5-FC treatment, MTT assays were carried out again and the final cell number as a proportion of the starting cell number was calculated. The results for the three concentrations of 5-FC (0.1, 1, 5 mM) are given as a proportion of the corresponding untreated (0 mM 5-FC) cells. The results were analysed by two-way ANOVA. Differences between means were tested for significance with Bonferroni post-hoc tests.

For LNCaP cells grown in air, there was no significant difference between the cell numbers of pPPE-CD or pCMV-CD compared to untransfected cells for any of the 5-FC concentrations given (Figure 4.16). The effect was more pronounced when the cells were grown in air with 1000 nM DHT, with a significant change in cell numbers for pPPE-CD compared to untransfected cells treated with 1 mM 5-FC ($p < 0.05$) (Figure 4.17). For pCMV-CD transfected cells compared to untransfected cells, the difference was significant for all three concentrations, 0.1 mM ($p < 0.05$), 1 mM ($p < 0.001$) and 5 mM 5-FC ($p < 0.05$). Growth with 0.5% oxygen, with or without DHT did not result in a significant decrease in cell number for pPPE-CD transfected LNCaP cells compared to untransfected cells for 0.1 mM, 1 mM or 5 mM 5-FC (Figure 4.18 and Figure 4.19). When grown in 0.5% oxygen with DHT before 5-FC treatment, cell survival was significantly lower for pCMV-CD transfected than untransfected cells for 0.1 mM ($p < 0.01$), 1 mM 5-FC ($p < 0.01$) and 5 mM 5-FC ($p < 0.05$).

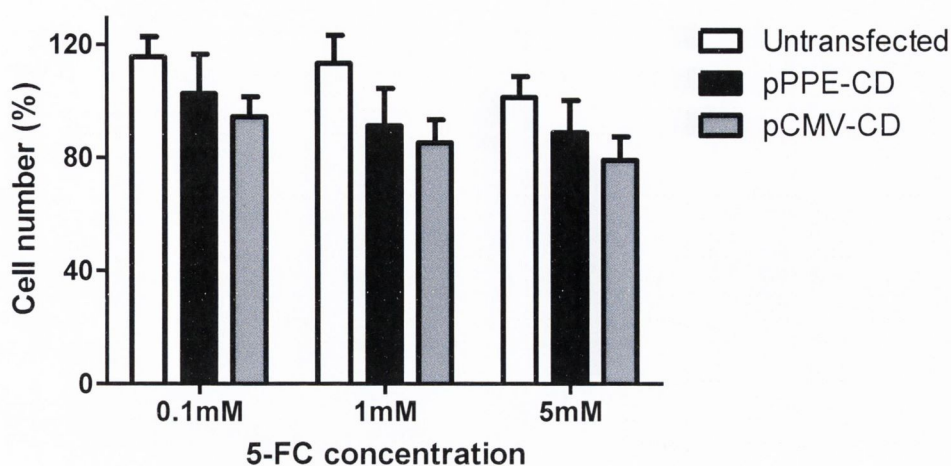


Figure 4.16: LNCaP cell count after treatment in air. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean.

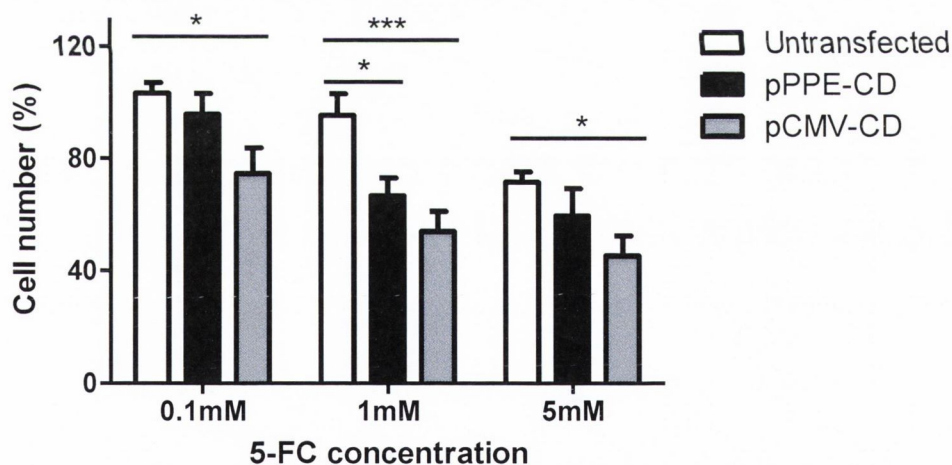


Figure 4.17: LNCaP cell count after treatment in air with 1000 nM DHT. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean. ***, $p < 0.001$; *, $p < 0.05$.

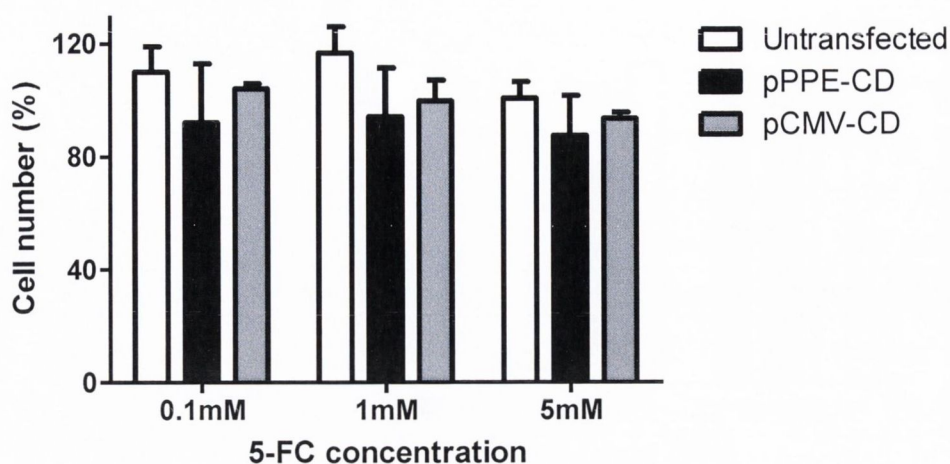


Figure 4.18: LNCaP cell count after treatment in hypoxia. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean.

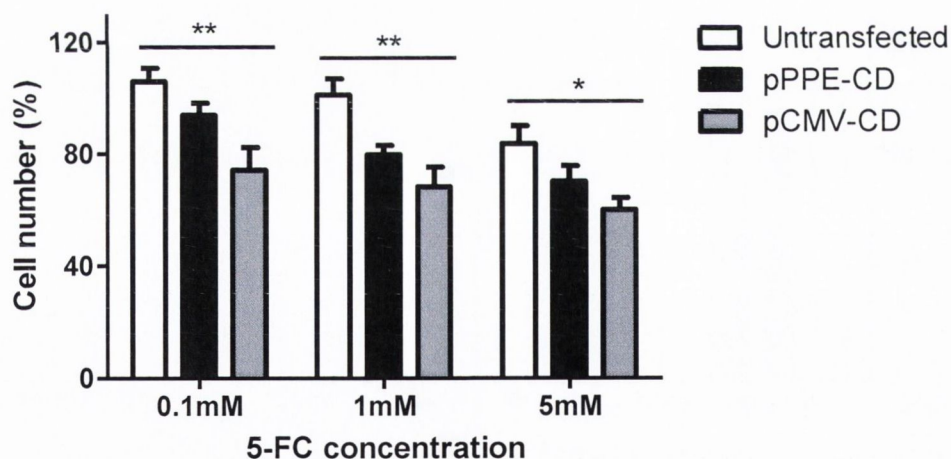


Figure 4.19: LNCaP cell count after treatment in hypoxia with 1000 nM DHT. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean. **, $p < 0.01$; *, $p < 0.05$.

For 22Rv1 cells grown in air for three days following transfection and then treated with various concentrations of 5-FC there was no significant difference between cell survival of the pPPE-CD transfected cells and untransfected cells for 0.1 mM, 1mM or 5 mM 5-FC (Figure 4.20). The difference between the pCMV-CD transfected cells and untransfected cells was highly significant for 0.1 mM ($p < 0.001$), 1 mM ($p < 0.001$) and 5 mM 5-FC ($p < 0.01$). When the cells were grown with 1000 nM DHT in air before 5-FC treatment, the number of cells was significantly less for the pPPE-CD transfected cells compared to untransfected cells for 1 mM and 5 mM 5-FC ($p < 0.05$) (Figure 4.21). For pCMV-CD transfected vs. untransfected cells, the difference was highly significant ($p < 0.001$) for the three concentrations.

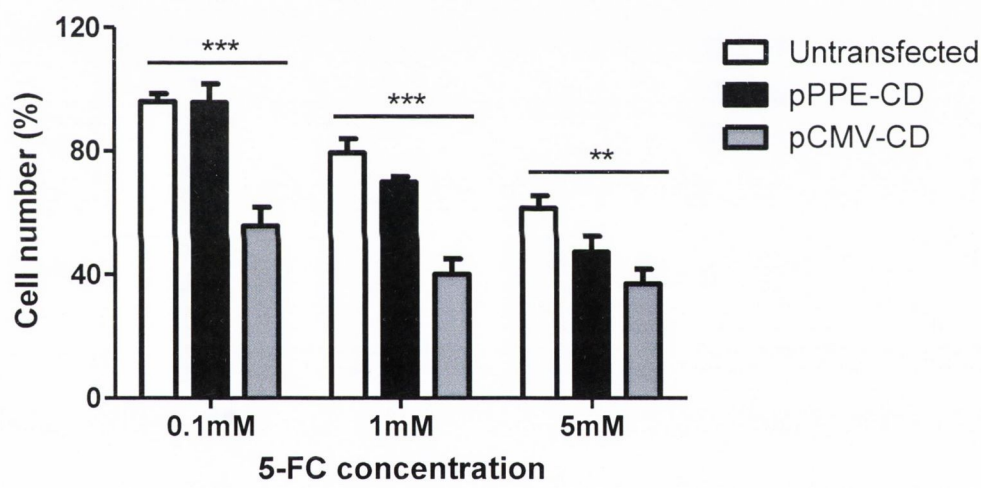


Figure 4.20: 22Rv1 cell count after treatment in air. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean. ***, $p < 0.001$; **, $p < 0.01$.

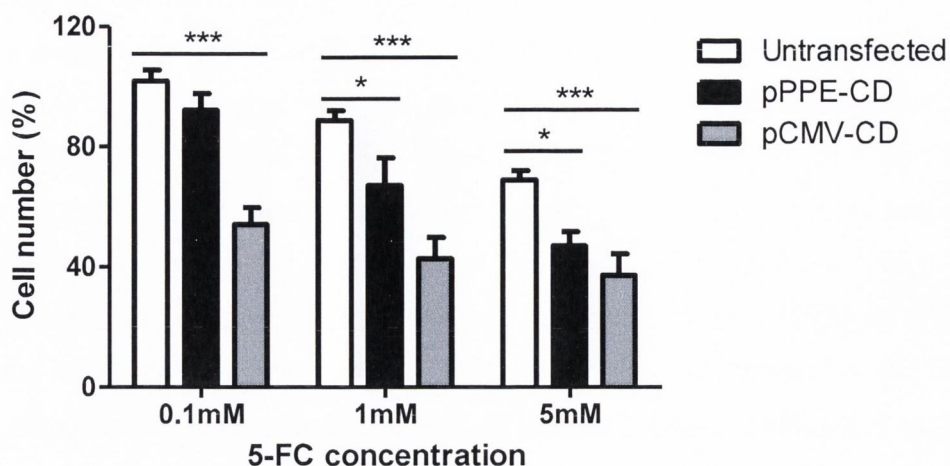


Figure 4.21: 22Rv1 cell count after treatment in air with 1000 nM DHT. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean. ***, $p < 0.001$; *, $p < 0.05$.

Growth under hypoxic conditions before 5-FC treatment did not result in a significant decrease in cell proliferation in pPPE-CD transfected cells compared to the untransfected control cells (Figure 4.22). There was a significant difference between pCMV-CD transfected 22Rv1 and the untransfected cells at 0.1 mM ($p < 0.001$) and 1 mM 5-FC ($p < 0.05$). When the cells were grown in 0.5% oxygen with 1000 nM DHT there were significantly less pPPE-CD transfected cells than untransfected cells for 1 mM 5-FC ($p < 0.001$) (Figure 4.23). Cell survival was significantly lower for pCMV-CD transfected than untransfected cells for 0.1 mM ($p < 0.001$) and 1 mM 5-FC ($p < 0.01$).

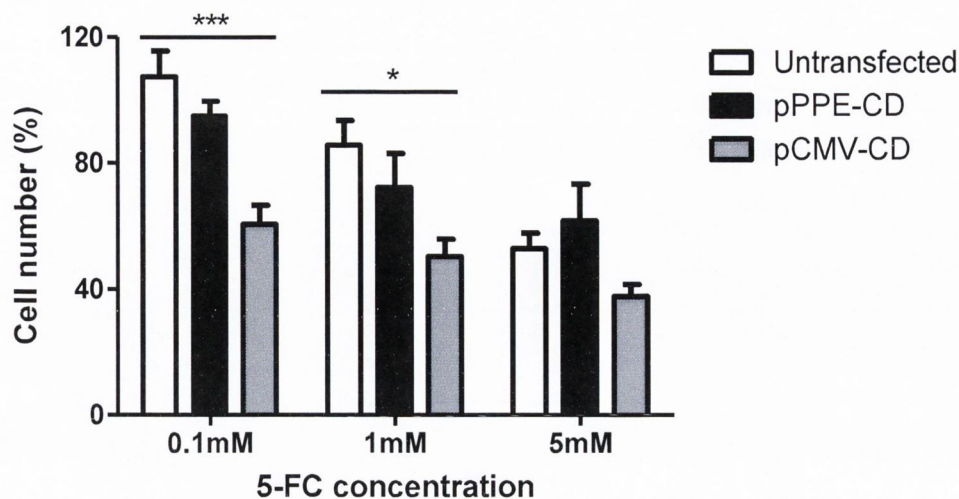


Figure 4.22: 22Rv1 cell count after treatment in hypoxia. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean. ***, $p < 0.001$; *, $p < 0.05$.

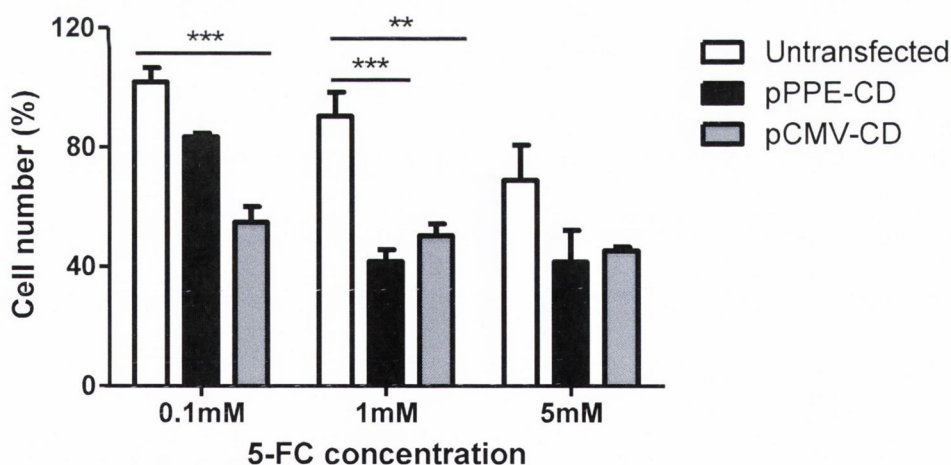


Figure 4.23: 22Rv1 cell count after treatment in hypoxia with 1000 nM DHT. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean. ***, $p < 0.001$; **, $p < 0.01$.

For those 22Rv1 cells grown with 1000 nM DHT before 5-FC treatment, the decrease in cell number was significantly lower for pPPE-CD transfected cells than untransfected at the clinically relevant dose of 1 mM 5-FC. This result was observed for cells treated in both air (21% oxygen) and hypoxia (0.5%) but the degree of significance was greater for the hypoxic cells ($p < 0.001$ vs. $p < 0.05$) indicating activity of pPPE-CD is improved when hypoxia and DHT stimulation are combined compared to DHT alone (Figure 4.24).

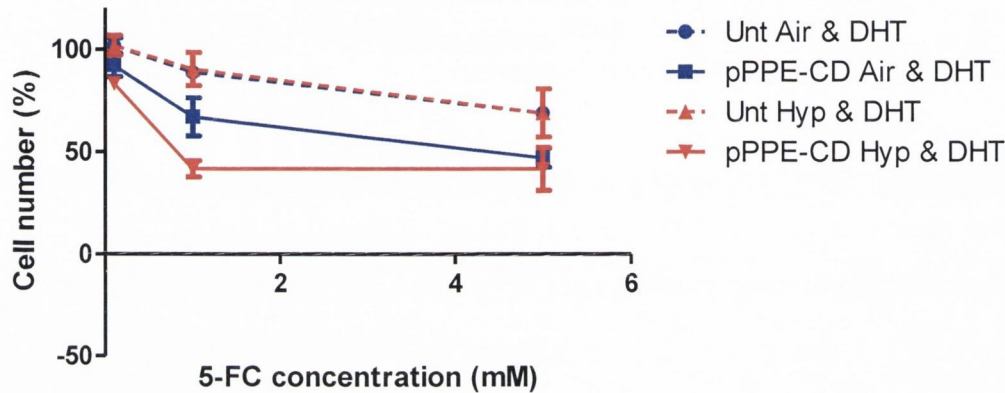


Figure 4.24: 22Rv1 cell numbers for untransfected (dashed line) and pPPE-CD transfected (solid line) cells grown in air (blue) or hypoxia (red) with 1000 nM DHT and treated with various concentrations of 5-FC. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three independent replicates $\pm$ standard error of the mean. Unt, untransfected; Hyp, hypoxia.

Clonogenics assay testing pPPE-CD/5-FC under aerobic and hypoxic conditions

Clonogenic assays were carried out to further test the toxicity of the pPPE-CD/5-FC system by assessing the colony forming ability of 22Rv1 cells transfected with pPPE-CD, pCMV-CD or mock transfected and grown in 21% or 0.5% oxygen with or without DHT for 72 hours. The media was then replaced with fresh CD-FBS media and 1 mM 5-FC was added to half the plates with the other half serving as the 0 mM 5-FC controls. After a further 72 hours the media was replaced again and the plates were incubated for 10-14 days before crystal violet staining. The results for each treatment condition were expressed as a percentage of the equivalent treatment

without 5-FC and were analysed using a two-way ANOVA and Bonferroni post-hoc tests. For those transfectants grown in air, 5-FC treatment resulted in a significant difference ($p < 0.05$) in the number of colonies for pPPE-CD-22Rv1 treated with DHT (Figure 4.25). When samples were grown under hypoxic conditions (0.5% oxygen), a significant decrease in colony number was observed for pPPE-CD transfectants grown in hypoxia alone but this was also the case for untransfected cells (Figure 4.26). There was also a significant decrease in colony number for the cells transfected with pCMV-CD with the addition of 5-FC.

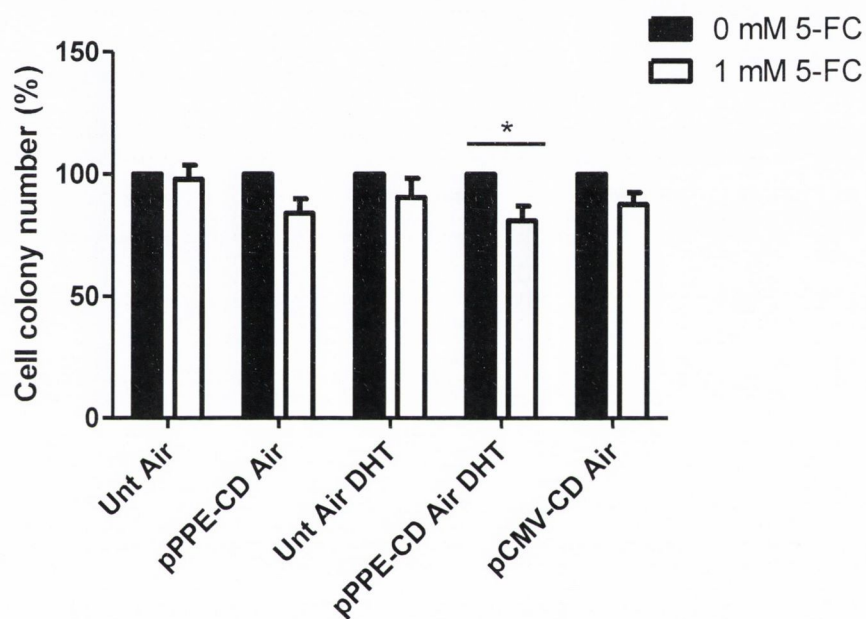


Figure 4.25: Cell colony number following clonogenic assays of 22Rv1 grown in air with or without DHT and treated with 5-FC. Unt, untransfected; *, $p < 0.05$. Values represent the mean of three independent replicates $\pm$ standard error of the mean.

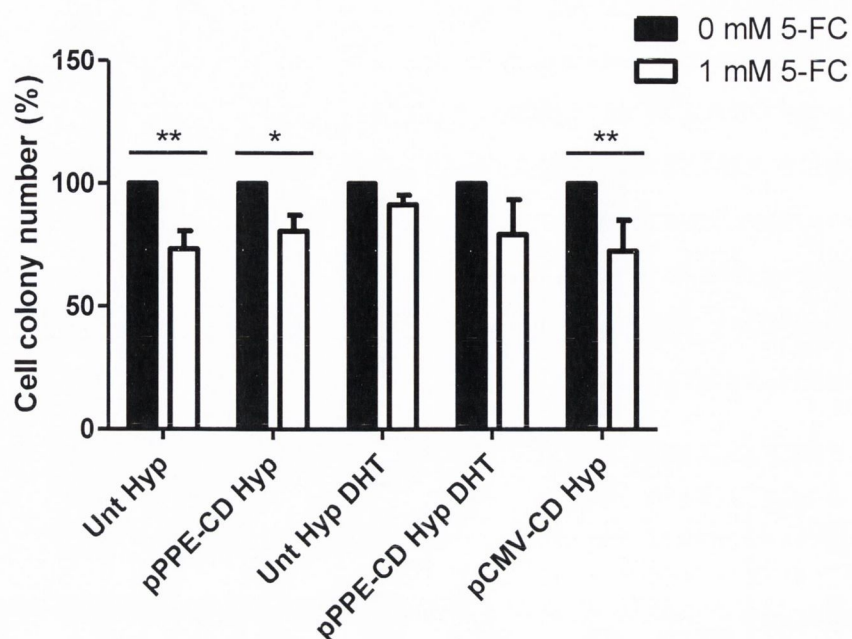


Figure 4.26: Cell colony number following clonogenic assays of 22Rv1 grown in hypoxia with or without DHT and treated with 5-FC. Unt, untransfected; *, $p < 0.05$; **, $p < 0.01$. Values represent the mean of three independent replicates $\pm$ standard error of the mean.

4.3 Discussion

This research involved a GDEPT vector previously created by Dr. Ruth Foley, pPPE-CD, in which the gene for the enzyme cytosine deaminase is under the control of the PSA promoter and enhancer. It was previously established that CD was expressed in pPPE-CD transfected cells following treatment with the androgen, DHT. This investigation tested the potential of the pPPE-CD vector system to be activated by hypoxia (0.5% oxygen) as well as androgen treatment and was based on recent research which has revealed the synergistic relationship between AR and HIF-1.^{190, 211, 212} This research has shown that growth under hypoxic conditions results in the further upregulation of androgen responsive genes. Of particular relevance to the current study was the previous research of the PSA gene promoter, demonstrating the importance of the ARE III and the presence of a hypoxia response element that allows for PSA stimulation by hypoxia alone.¹⁹⁰

In light of these findings, the research detailed in this chapter aimed to determine if the pPPE-CD/5-FC gene therapy system had potential to target hypoxic cells as well as androgen-positive cells. This is relevant to prostate cancer as hypoxia is a common characteristic, as well as being observed in other solid tumours.²¹⁵ Hypoxia results in the upregulation of genes that control angiogenesis and glycolysis, which is associated with tumour aggressiveness and metastasis.^{44, 51} It is associated with poor prognosis and can lead to resistance to both chemotherapy and radiation.²¹⁶

Through initial Western blotting, it was demonstrated that CD expression in pPPE-CD transfected cells could be increased if growth in hypoxia (0.5% oxygen) was combined with DHT in both LNCaP and 22Rv1 cells compared to DHT alone and that hypoxia alone was capable of inducing CD in 22Rv1. This was encouraging evidence that the AR/HIF-1 cross-talk was capable of influencing the PSA promoter/enhancer in the pPPE-CD vector. This led to the study of cell kill when pPPE-CD transfection and DHT/hypoxia treatment were combined with the prodrug 5-FC using MTT assays. The results for the 5-FC treated samples were normalised to the 0 mM 5-FC sample for each treatment condition so that only the changes in cell number resulting from 5-FC treatment would be measured, controlling for variation in cell proliferation resulting from variables such as growth in hypoxia or treatment with DHT.

The assays demonstrated the efficacy of this system to reduce cell viability although the results were more marked in 22Rv1 cells than LNCaP. For 22Rv1 cells, growth in air (21% oxygen) or hypoxia (0.5%) alone did not result in any significant difference between the pPPE-CD transfected cells and the untransfected cells at any of the 5-FC concentrations. For DHT stimulation under aerobic conditions, there was a significant decrease in cell number ($p < 0.05$) for pPPE-CD transfected 22Rv1 compared to untransfected cells for 1 mM and 5 mM. This confirmed that androgen stimulation of CD expression in transfected cells results in toxicity when combined with prodrug treatment. When hypoxia and DHT treatment were combined the decrease in cell number for 1 mM 5-FC was even more pronounced ($p < 0.001$) suggesting that the increase in CD expression from PPE-CD transfected cells when DHT is combined with hypoxia has the potential to further increase toxicity at this dose of 5-FC. In LNCaP cells transfected with pPPE-CD compared to the untransfected control cells, there was a significant decrease in cell survival when 1 mM 5-FC was used. Under hypoxic conditions, with or without DHT, the decrease in cell numbers for pPPE-CD transfectants compared to untransfected cells did not reach significance.

1 mM is the most clinically relevant of the doses of 5-FC used. A less pronounced difference between pPPE-CD transfected and untransfected cells was observed for 5 mM 5-FC under the various treatment conditions. Although 5-FC is a prodrug of relatively low toxicity, 5 mM concentrations were toxic to untransfected cells. 1 mM 5-FC was selected for further testing using clonogenic assays. LNCaP cells are not suitable for use in clonogenic assays but as 22Rv1 cells showed a more pronounced response to this treatment strategy, it was decided to proceed with this technique in 22Rv1 cells only.

For those cells grown in 21% oxygen, a significant decrease in colony number with 5-FC treatment was only observed in the pPPE-CD transfected cells grown with DHT (Figure 4.25). This indicates that 5-FC toxicity is not observed for the untransfected cells and that DHT stimulation is necessary for pPPE-CD activation. Under hypoxic (0.5% oxygen) conditions, a significant decrease in colony forming ability was observed for both the pPPE-CD transfected cells and the untransfected cells (Figure 4.26). This is a curious result that suggests 5-FC can reduce colony

numbers in the absence of CD expression. Further clonogenic assay experiments were carried out to compare the effect of 5-FC treatment in air and hypoxia (Appendix G). 22Rv1 and DU145 cells were treated with 1 mM and 5 mM 5-FC under aerobic and hypoxic conditions for 48 and 72 hours. For the 1 mM 5-FC concentration there was a significant decrease in 22Rv1 colony forming ability for those cells grown in hypoxia compared to air ($p < 0.05$ for 48 hours treatment and $p < 0.01$ for 72 hours treatment). This significant difference between the effect of 5-FC in air and hypoxia was not observed for DU145 cells. Also, the effect was not seen in the previous MTT analysis of pPPE-CD activity. It is unclear why, in the clonogenic assays, 5-FC treatment should be toxic under hypoxic conditions in 22Rv1 cells. It is a result which warrants further investigation but if genuine, is an encouraging result, as any improvement of pPPE-CD/5-FC efficacy would be beneficial.

The clonogenic assays did not show the same increase in pPPE-CD efficacy in hypoxia (0.5% oxygen) compared to air (21%) that was previously observed for the MTT assays. The MTT assay assesses the number of viable cells by testing their ability to convert a tetrazolium base compound to a blue formazan compound whereas the clonogenic assay is a test of the colony forming ability of the cells following treatment. The clinically relevant dose of 1 mM 5-FC showed potential following the MTT assays and was chosen for use in the clonogenic assays over the other doses (0.1 mM and 5 mM). It is possible however, that this dose was not sufficient to observe an effect when clonogenic assays were carried out.

This work highlighted some of the technical difficulties of working with the LNCaP cell line. Before DHT treatment, it is necessary to grow the cells in RPMI media containing FBS that has been charcoal/dextran treated to reduce the levels of hormones, steroids and growth factors. In the initial experiment to test efficacy of pPPE-CD/5-FC treatment, an MTT assay was carried out on sample wells after growth in the hormone-free media but before hypoxia/DHT treatment and then the absorbance was measured for all the wells at the end of the experiment. For some of the LNCaP samples, the absorbance readings taken at the end were lower than those original readings, indicating cell death, not just a decrease in proliferation. This occurred for samples grown in 21% oxygen without DHT and those grown in 0.5%

oxygen with or without DHT, even for those not treated with 5-FC. This toxicity may have reduced the impact of 5-FC treatment and confounded interpretation of the results.

There is a strong evidence for the presence of hypoxic regions in a host of solid tumours. HIF-1 α overexpression has been detected in a range of tumour types, including prostate, compared to normal tissue.²¹⁷ The presence of hypoxia has been shown to have a negative impact on treatment outcome for those receiving radical prostatectomy as well as those given radiation treatment.²¹⁶ Radiation therapy is negatively affected by hypoxia, as oxygen can make the damage caused by ionizing radiation permanent by oxidizing the free radical that forms on the DNA.⁴⁹ On top of that, hypoxia, more specifically HIF-1, upregulates a large number of genes that allow for enhanced survival, invasion and aggressiveness of the tumour. When treatment failure is so strongly linked to hypoxia, it is logical to target these regions of the tumour. Low levels of oxygen are not generally observed in normal tissue, allowing for a treatment strategy that is tumour-specific. The pPPE-CD vector shows potential in that regard. pPPE-CD contains the enzyme CD under the control of the PSA promoter and enhancer, which is regulated by the AR. PSA is expressed exclusively in the epithelial cells of the prostate¹⁶⁸ and when pPPE-CD was developed, the initial goal was to achieve androgen-inducible activity. Research has demonstrated a cross-talk between HIF-1 and AR signalling.^{190, 211, 212} The exact details of the mechanism controlling this has yet to be elucidated but it was found that the PSA gene has a hypoxia response element in its promoter as well as a number of androgen response elements.¹⁹⁷ In LNCaP cells, PSA expression is upregulated by a greater extent when hypoxia and DHT stimulation are combined.¹⁹⁷ This led to the hypothesis that pPPE-CD may be hypoxia as well as androgen responsive. The research described in this chapter demonstrated that that is indeed the case, with higher levels of CD expression under hypoxic conditions and MTT assays showed a resulting decrease in cell number when hypoxia and DHT treatments are combined compared to DHT alone (although this only reached significance in 22Rv1). pPPE-CD has potential as a prostate-specific gene therapy vector which has highest activity in the hypoxic regions of the tumour which are the ones most likely to show resistance to current treatment methods.

The two cell lines used as models in which to test this GDEPT system are a primary cancer line, 22Rv1 and a metastatic one, LNCaP. Both cell lines are known to be AR positive and to secrete PSA.^{218, 219} LNCaP cells are more typically used in experiments involving the PSA promoter but 22Rv1 cells serve as one of the few models of primary prostate cancer, and pPPE-CD may have potential as an adjuvant given alongside current treatment options for this stage of the disease. Initial experiments testing PSA protein expression levels in these cell lines in response to DHT stimulation in 21% and 0.5% oxygen demonstrated an obvious increase in PSA expression levels in LNCaP cells as result of the cross-talk between AR and HIF-1 (Figure 4.4). In 22Rv1 cells, however, the PSA levels were significantly lower than for LNCaP and there was no increase in protein expression in hypoxic compared to aerobic cells (Figure 4.5). This initial evidence suggested 22Rv1 cells may not have been a suitable model in which to test the interaction between DHT and hypoxia stimulation of a vector in which the PSA promoter controls expression. However, when 22Rv1 cells were transfected with pPPE-CD and treated with DHT in 21% and 0.5% oxygen, CD protein expression was strong and hypoxia was shown to increase levels of CD, as it did in LNCaP. This revealed that 22Rv1 cells were in fact a suitable cell line, alongside LNCaP, in which to characterise the activity of pPPE-CD. Later work in Chapter 6 involves attempts to modify the 22Rv1 cell line towards higher PSA expression to improve it as a model in which to test the efficacy of pPPE-CD.

To the author's knowledge, this is the first time that cross talk between androgen stimulation and hypoxia has been shown to affect gene expression in 22Rv1 cells. The contrast between PSA and CD expression in the 22Rv1 cell line is interesting. The observed influence of cross-talk on CD expression indicates that the AR and HIF-1 machinery are capable of upregulating androgen-responsive gene expression in 22Rv1 cells, as has previously been demonstrated in LNCaP. It is possible that the differences between the endogenous PSA promoter and associated regulatory elements in 22Rv1 and the one used in the pPPE-CD vector are responsible for the very different levels of protein expression and response to hypoxic treatment.

The work by *Horii et al.* puts forth evidence for the presence of a hypoxia response element in the PSA promoter and a physical interaction between AR and HIF-1 α

when they bind to the HRE and ARE III. The HRE sequence, 5'-RCGTG-3', is present in the PSA promoter sequence of pPPE-CD, as is the ARE III, with one bp change. It is possible that in the PSA promoter of 22Rv1 there is a mutation in the HRE or ARE III, which results in low PSA expression and also prevents cross-talk. Mitani *et al.* showed that the expression of another androgen responsive gene, NKX3.1, is increased in LNCaP when DHT treatment is given in hypoxia compared to air.²¹² Growth in hypoxia, without DHT, was not sufficient to upregulate expression, in contrast to PSA. It would be informative to look at NKX3.1 expression in 22Rv1 cells and determine if the AR/HIF-1 cross-talk influences expression in the same way as in LNCaP cells. If so, this would indicate a mutation in the PSA promoter or enhancer within 22Rv1 cells which is preventing the upregulation of expression under hypoxic conditions. Future work could involve sequencing the PSA promoter and enhancer of 22Rv1 to compare it to the wild-type sequence to shed more light on the sequences necessary for AR/HIF-1 cross-talk activity.

Chapter 5

Investigation of pPPE-CD activity in
combination with radiation

Chapter 5 Investigation of pPPE-CD activity in combination with radiation

5.1 Introduction

The potential of the pPPE-CD/5-FU system as an adjuvant to radiation therapy was investigated as part of this research. Radiation therapy is one of the main treatment strategies, alongside radical prostatectomy, given to patients with localised prostate cancer. Prognosis for patients with localised disease is relatively good, especially for those with low-risk disease (~80% following radiation therapy). For those with intermediate or high-risk forms of the disease the rates are lower (25-75%).¹⁵⁸ It is logical to try to develop treatment strategies that improve patient outcome at this early stage of the disease as curative options for those with late stage disease are limited.

Technological advances in recent years have allowed for the administration of higher doses of radiation in an attempt to improve the efficacy of radiation therapy.⁶⁰ Although successful, this strategy is somewhat limited by the risk of increased side-effects. Another way to improve treatment outcome may be to administer additional therapies alongside radiation treatment. Currently the available hormonal neoadjuvant and adjuvant treatments target AR: anti-androgens block AR or luteinizing hormone releasing hormone agonists reduce the amount of androgen.^{95, 96} Previous research has demonstrated the potential of GDEPT to work synergistically with radiation therapy. 5-FU has been shown to be a moderate radiosensitizer with evidence that it causes the inhibition of thymidylate synthase which stops DNA repair.^{141, 142} It is possible that a GDEPT treatment strategy given with radiation therapy could improve efficacy of this therapy.

In addition, research has been carried out using radiation-responsive GDEPT systems. As discussed in section 1.10.3, in response to radiation treatment, transcription factors activate the expression of a number of genes such as *Egr-1*, *NF-κB* and *p21^{WAF1/CIP1}*.¹⁸⁸ This has been exploited in GDEPT research to control transgene expression and achieve targeted therapy.¹⁸⁴ Radiation may induce gene expression indirectly also as *NF-κB*, for example, controls transcription of a host of other genes. These include AR and PSA, which is of relevance to this research

project.^{220, 221} In light of this, experiments were carried out to examine whether radiation had an effect on pPPE-CD activity and influenced CD expression levels.

5.1.1 Aims and objectives

The pPPE-CD/5-FC GDEPT system could have clinical potential in the treatment of prostate cancer as an adjuvant to radiation therapy.

Aim: To determine if radiation has an effect on CD expression.

Aim: To determine the effects of a combined treatment of clinically-relevant doses of radiation and 5-FC in pPPE-CD-transfected cells.

5.2 Results

5.2.1 *PSA expression in response to radiation*

Before combining the pPPE-CD/5-FC system with radiation treatment, PSA protein levels following irradiation were measured by EIA to assess whether this had any effect on expression. LNCaP and 22Rv1 cells were treated with a range of concentrations of DHT under aerobic (21% oxygen) or hypoxic conditions (0.5%). Two hours later, 2 Gy radiation was administered. After 72 hours, the PSA secretion levels were measured and it was found that there was no significant difference between the irradiated and unirradiated samples grown under aerobic or hypoxic conditions for LNCaP (Figure 5.1) or 22Rv1 (Figure 5.2).

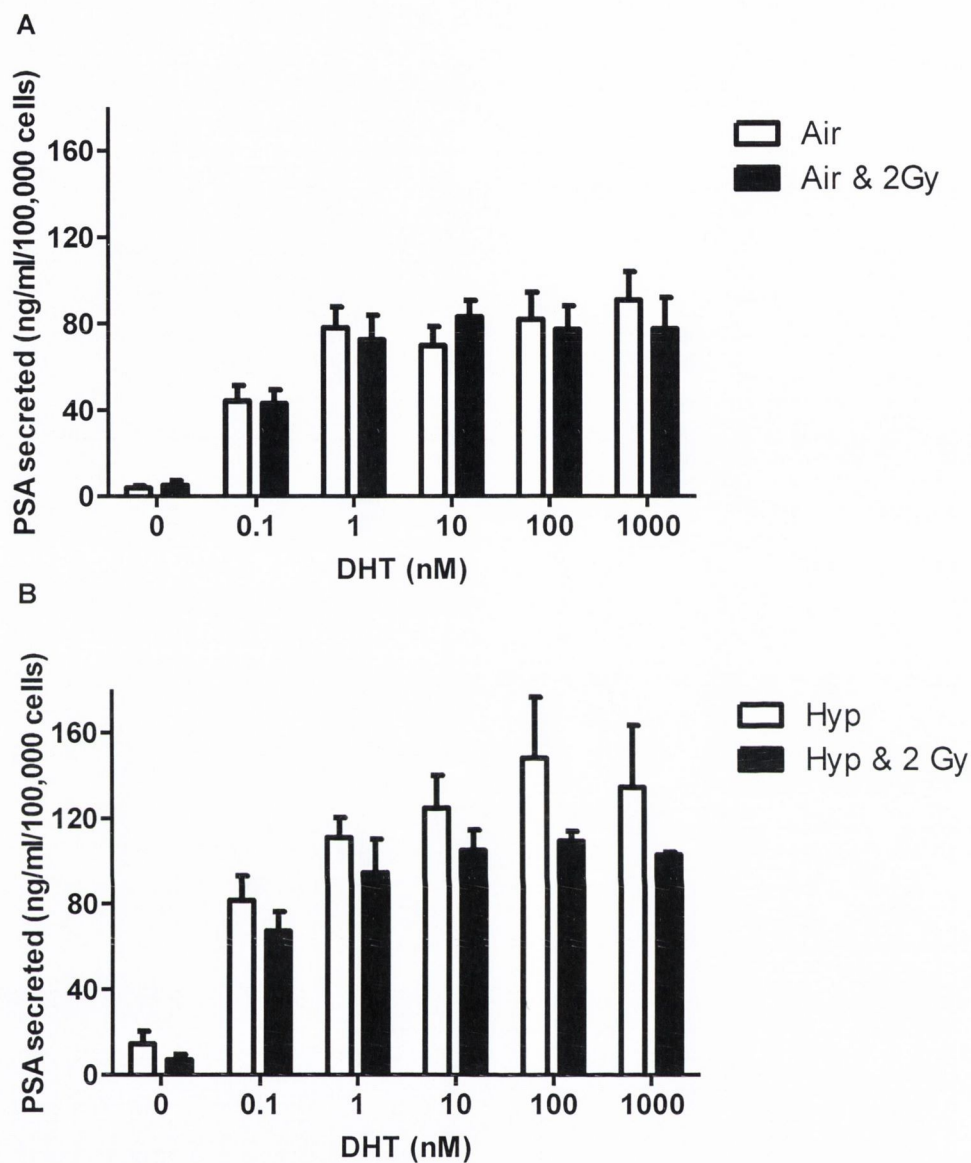


Figure 5.1: PSA protein levels measured by PSA EIA following DHT stimulation with or without irradiation in LNCaP under aerobic (A) and hypoxic conditions (B). Hyp, hypoxia. Values represent the mean of three independent replicates $\pm$ standard error of the mean.

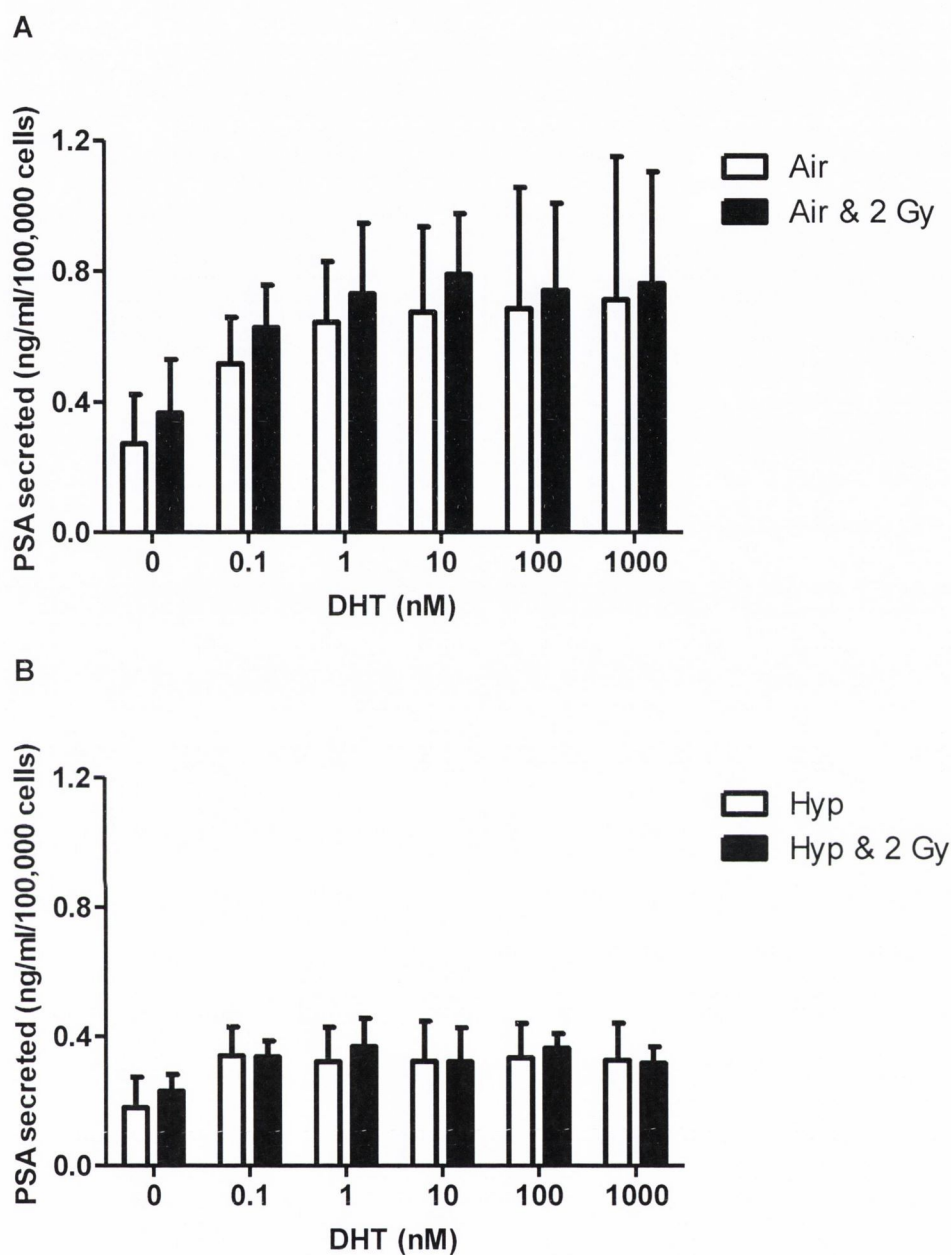


Figure 5.2: PSA protein levels measured by PSA EIA following DHT stimulation with or without irradiation in 22Rv1 under aerobic (A) and hypoxic conditions (B). Hyp, hypoxia. Values represent the mean of three independent replicates $\pm$ standard error of the mean.

The effect of radiation treatment on PSA was further analysed using qRT-PCR. In this experiment LNCaP and 22Rv1 cells were grown in 21% (air) or 0.5% (hypoxia) oxygen and treated with different doses of radiation (2, 6 or 8 Gy) before harvesting RNA. In LNCaP cells, PSA expression was significantly increased for aerobic samples when they were treated with radiation at all three doses (Figure 5.3). This difference in expression was further increased when irradiated cells were grown in hypoxia compared to the untreated aerobic cells. This effect on PSA gene expression is in contrast to the PSA EIA results, in which there was no significant difference between irradiated and unirradiated samples grown under either aerobic or hypoxic conditions. The trend observed for PSA expression levels in LNCaP cells in qRT-PCR analysis was not seen for 22Rv1 cells, with no significant difference between irradiated and non-irradiated samples (Figure 5.4).

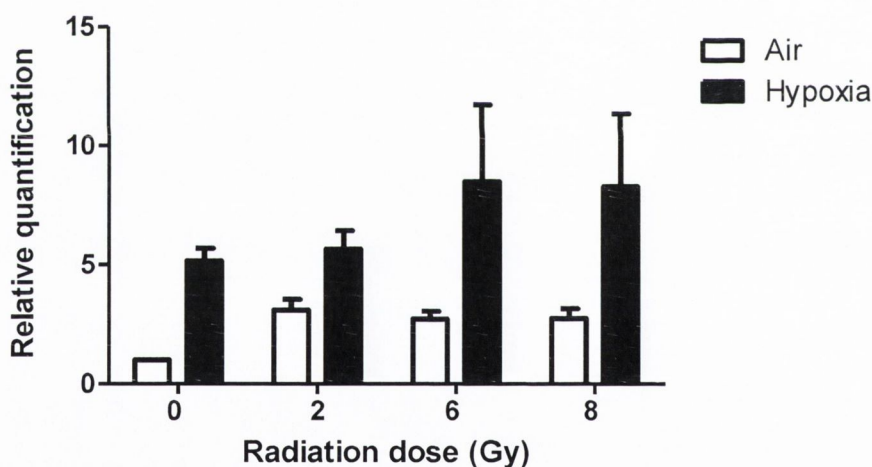


Figure 5.3: Relative quantification of *PSA* mRNA in aerobic and hypoxic LNCaP samples treated with various doses of radiation. Values represent the mean of three replicates $\pm$ standard error of the mean.

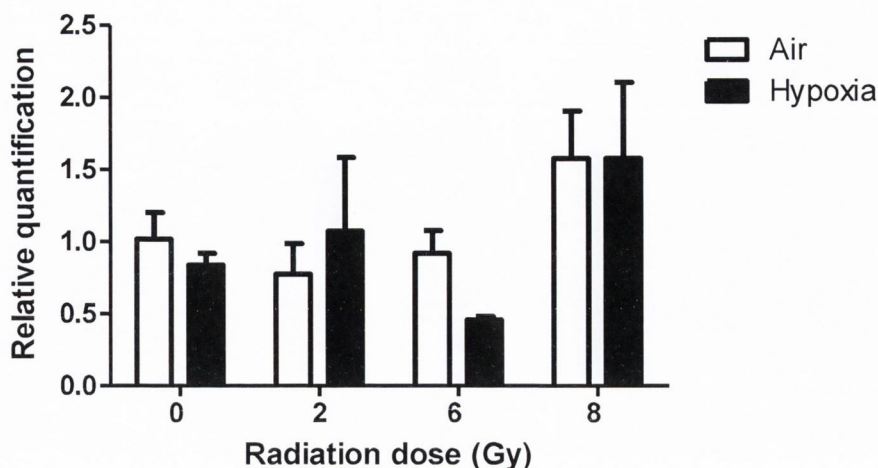


Figure 5.4: Relative quantification of *PSA* mRNA in aerobic and hypoxic 22Rv1 samples treated with various doses of radiation. Values represent the mean of three replicates $\pm$ standard error of the mean.

5.2.2 *pPPE-CD expression in response to radiation*

LNCaP and 22Rv1 cells were transfected with pPPE-CD, pCMV-CD or mock transfected with TransFast alone and then treated with a combination of DHT and radiation (2 Gy) in air (21% oxygen) and hypoxia (0.5%). In contrast to the PSA protein levels, Western blotting showed CD protein expression was higher for pPPE-CD transfected cells that had been irradiated and grown with 1000 nM DHT compared to cells treated with DHT alone. This was the case for both LNCaP (Figure 5.5 and Figure 5.6) and 22Rv1 (

Figure 5.7 and Figure 5.8).

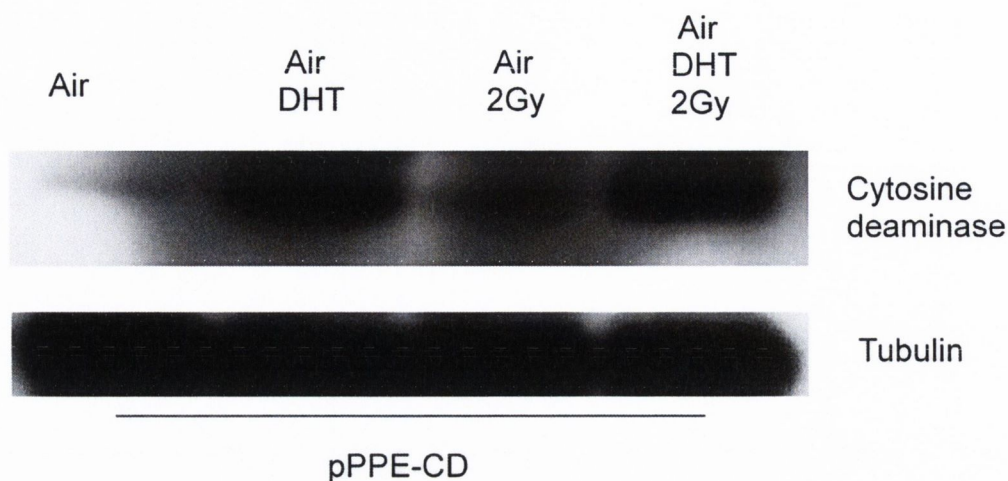


Figure 5.5: Cytosine deaminase protein expression in pPPE-CD transfected and untransfected LNCaP cells grown in air. Tubulin levels were used as a loading control. Representative blot, N=2. Hyp, hypoxia; DHT, 1000nM DHT; 2 Gy, 2 Gy radiation.

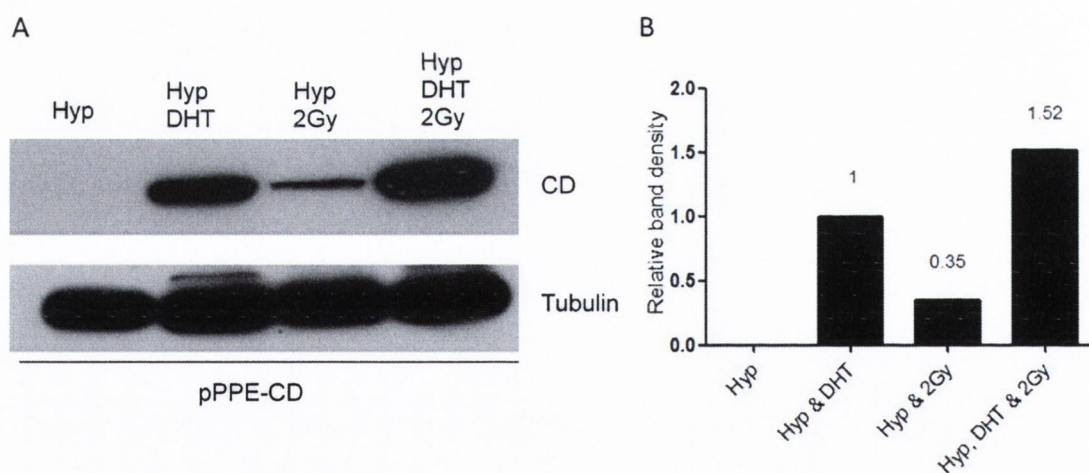


Figure 5.6: A) Cytosine deaminase protein expression in pPPE-CD transfected LNCaP treated with DHT and/or 2 Gy under hypoxic conditions. Tubulin levels were used as a loading control. Representative blot, N=2. Hyp, hypoxia; DHT, 1000nM DHT; 2 Gy, 2 Gy radiation. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

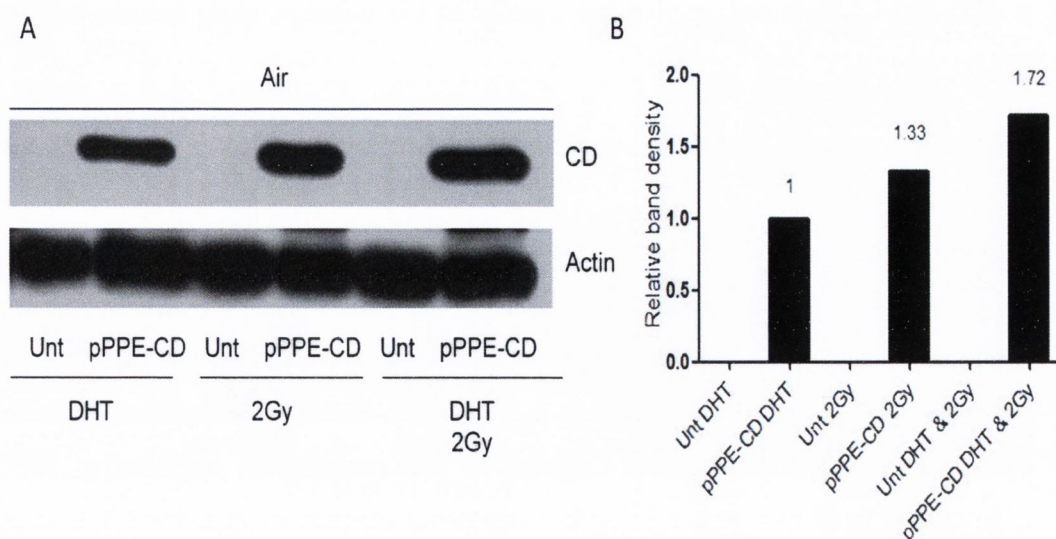


Figure 5.7: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected 22Rv1 cells grown in air. Tubulin levels were used as a loading control. Representative blot, N=2. Unt, untransfected; Hyp, hypoxia; DHT, 1000nM DHT; 2 Gy, 2 Gy radiation. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

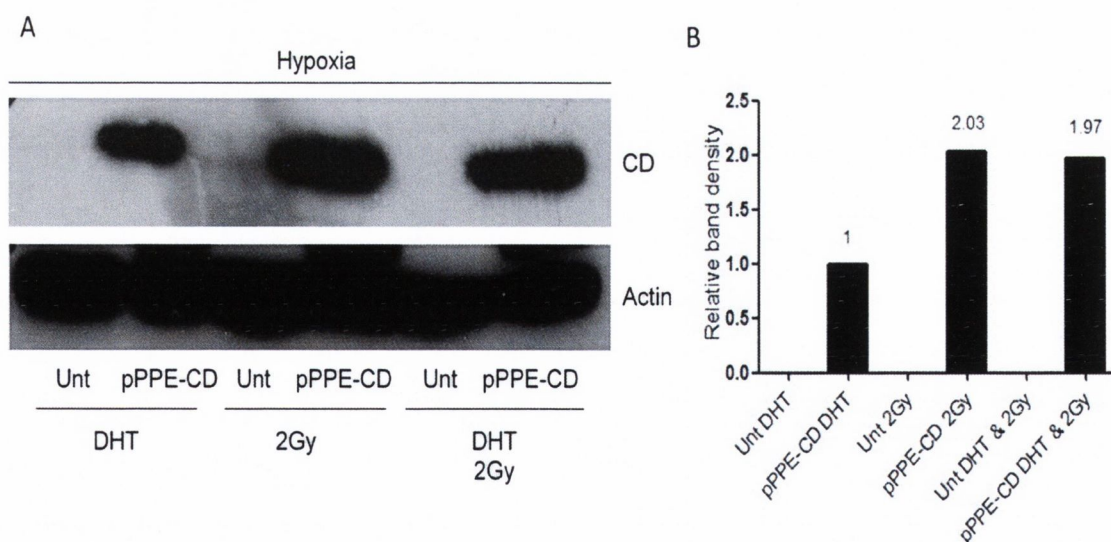


Figure 5.8: A) Cytosine deaminase protein expression in pPPE-CD transfected and untransfected 22Rv1 cells grown in hypoxia. Tubulin levels were used as a loading control. Representative blot, N=2. Unt, untransfected; Hyp, hypoxia; DHT, 1000nM DHT; 2 Gy, 2 Gy radiation. B) Densitometry analysis of the relative density of the

protein bands, corrected against the density of the corresponding loading control band.

5.2.3 Cell viability in pPPE-CD transfected LNCaP and 22Rv1 with radiation treatment

Western blotting results demonstrated that there was an increase in CD expression in pPPE-CD transfected LNCaP and 22Rv1 cells when radiation treatment was combined with androgen stimulation compared to androgen alone. Following on from this, MTT assays were carried out to check if this combination treatment resulted in a more pronounced decrease in cell numbers in response to 5-FC treatment. The assays were carried out following procedures described in section 4.2.2. Cells were grown with or without DHT in aerobic (21% oxygen) and hypoxic (0.5%) conditions and were treated with 2 Gy radiation before 5-FC treatment. The results for the three concentrations of 5-FC (0.1, 1, 5 mM) are given as a proportion of the corresponding untreated (0 mM 5-FC) cells. The results were analysed by two-way ANOVA. Differences between means were tested for significance with Bonferroni post-hoc tests.

For LNCaP cells grown in air and treated with radiation, the decrease in cell numbers in pPPE-CD transfected cells compared to untransfected cells did not reach significance (Figure 5.9). This was also the case for cells grown in air with DHT and treated with 2 Gy (Figure 5.10). The change in cell number between pCMV-CD transfected LNCaP and untransfected LNCaP was significant for 0.1 mM ($p < 0.01$), 1 mM ($p < 0.01$) and 5 mM ($p < 0.05$) 5-FC. For cells grown in 0.5% oxygen either with or without DHT and treated with 2 Gy radiation, there was no significant difference between the cell numbers of the pPPE-CD or pCMV-CD compared to untransfected cells (Figure 5.11 and Figure 5.12).

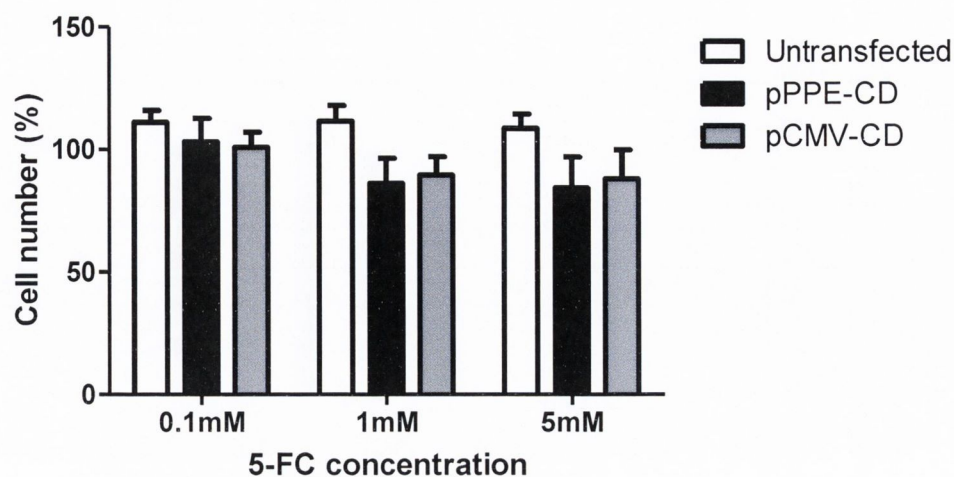


Figure 5.9: LNCaP cell count after treatment in air and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean.

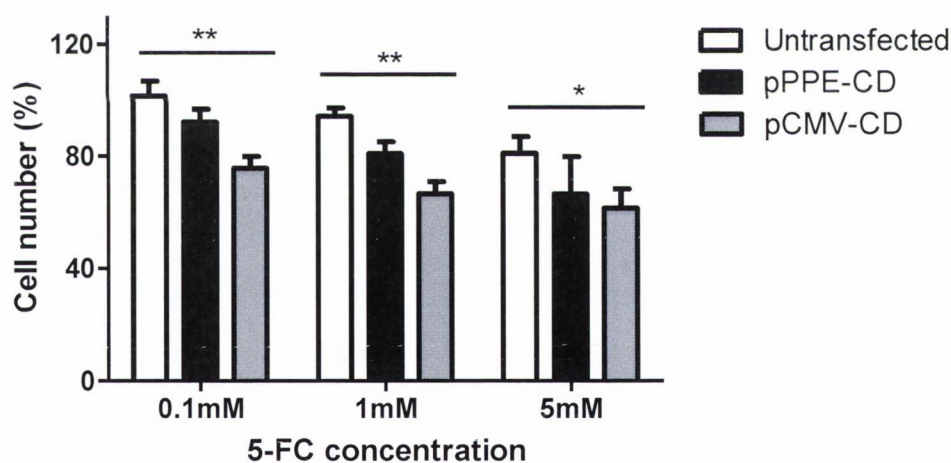


Figure 5.10: LNCaP cell count after treatment in air with 1000 nM DHT and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean. **, $p < 0.01$; *, $p < 0.05$.

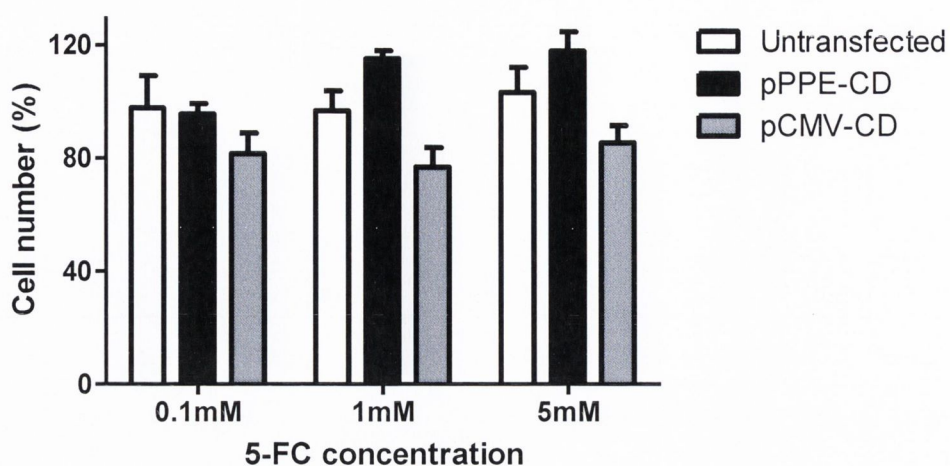


Figure 5.11: LNCaP cell count after treatment in hypoxia and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean.

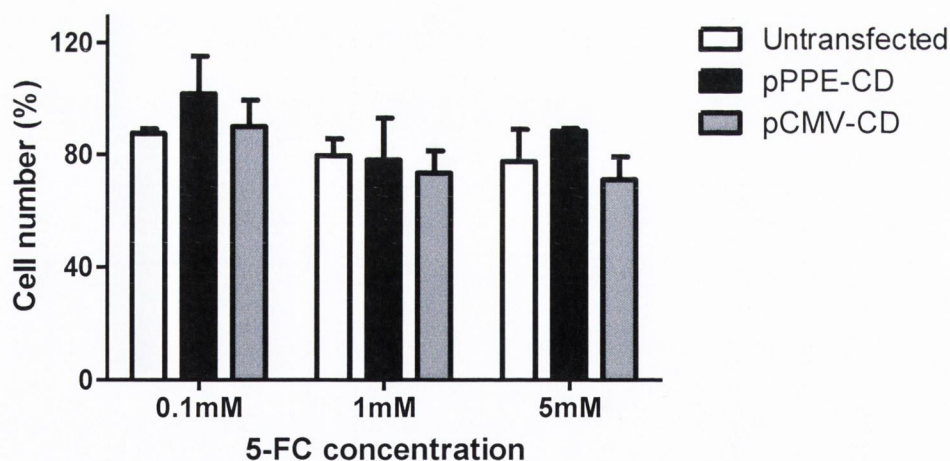


Figure 5.12: LNCaP cell count after treatment in hypoxia with 1000 nM DHT and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean.

For 22Rv1 cells there was no significant difference between the cell numbers for pPPE-CD transfected cells and untransfected cells when they were grown in air and treated with 2 Gy radiation before adding 5-FC (Figure 5.13). The difference between pCMV-CD transfected 22Rv1 and untransfected 22Rv1 was significant for 0.1 mM ($p < 0.001$), 1 mM ($p < 0.001$) and 5 mM ($p < 0.01$) 5-FC.

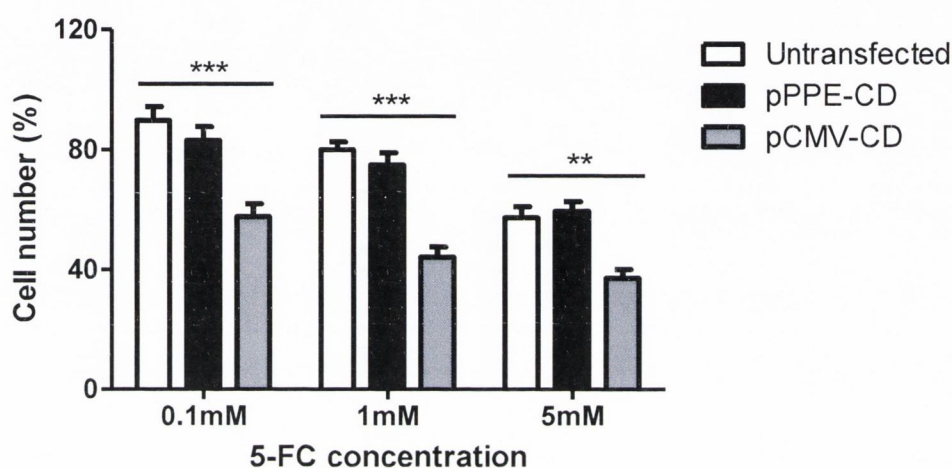


Figure 5.13: 22Rv1 cell count after treatment in air and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean. ***, $p < 0.001$; **, $p < 0.01$.

When the treatment of 22Rv1 cells with 2 Gy of radiation in air was combined with 1000 nM DHT the level of cell survival was significantly lower for those cells transfected with pPPE-CD and given 1 mM ($p < 0.01$) or 5 mM 5-FC ($p < 0.05$) compared to untransfected cells grown under the same conditions (Figure 5.14). The rates of cell survival were significantly lower for pCMV-CD transfected cells than untransfected cells for all three concentrations of 5-FC: 0.1 mM ($p < 0.001$), 1 mM ($p < 0.001$), 5 mM 5-FC ($p < 0.01$).

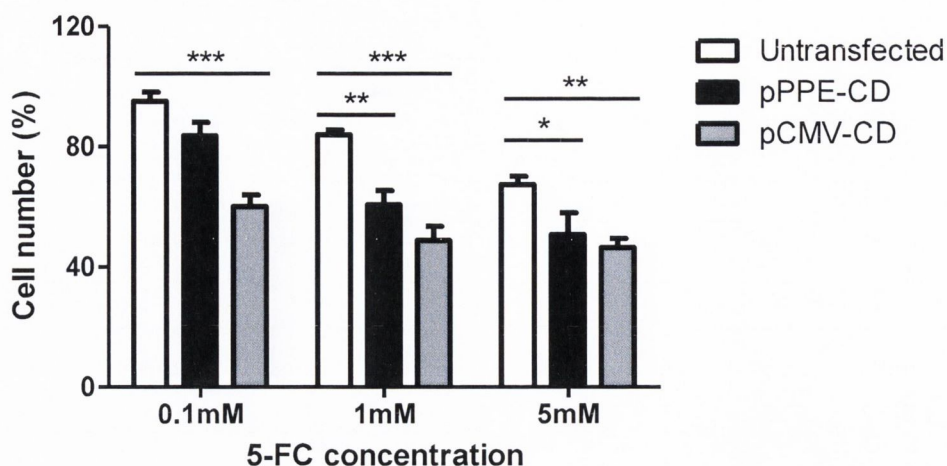


Figure 5.14: 22Rv1 cell count after treatment in air with 1000 nM DHT and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

For 22Rv1 cells grown in 0.5% oxygen and irradiated with one dose of 2 Gy the difference in cell survival between pPPE-CD transfected and untransfected cells did not reach significance (Figure 5.15). There was a significant difference ($p < 0.01$) between pCMV-CD transfected 22Rv1 and untransfected cells at 0.1 mM and 1 mM 5-FC. When the cells were grown with 1000 nM DHT in hypoxia and treated with 2 Gy of radiation, Bonferroni post-hoc testing showed there was a significance difference between cell numbers for pPPE-CD transfected 22Rv1 and untransfected 22Rv1 ($P < 0.01$) (Figure 5.16).

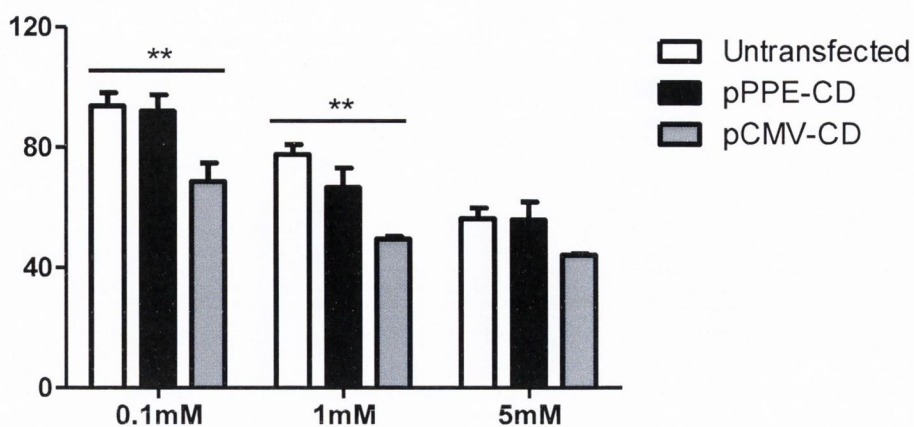


Figure 5.15: 22Rv1 cell count after treatment in hypoxia and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean. **, $p < 0.01$.

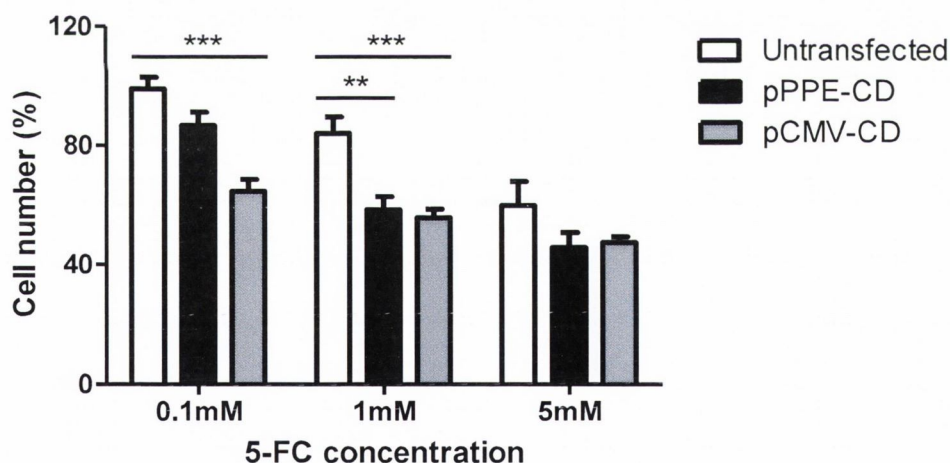


Figure 5.16: 22Rv1 cell count after treatment in hypoxia with 1000 nM DHT and irradiation with 2 Gy. Results are expressed as a proportion of the 0 mM 5-FC control. Values represent the mean of at least three replicates $\pm$ standard error of the mean. ***, $p < 0.001$; **, $p < 0.01$.

The results for 22Rv1 cells grown in air were compared to the earlier findings for pPPE-CD/5-FC treatment without radiation (section 4.2.2) using a one way

ANOVA and Bonferroni multiple comparison test. For pPPE-CD transfected 22Rv1supplemented with DHT and treated with radiation, cell number decreased but the change was not significant (

Figure 5.17). The decrease in cell number for those cells treated with 1mM 5-FC was significant ($p < 0.05$). Importantly, when DHT, 2 Gy radiation and 1 mM 5-FC were combined the cell number was significant lower compared to pPPE-CD DHT alone ($p < 0.001$). This suggests there may be an improvement in pPPE-CD/5-FC toxicity when the treatment is given together with radiation.

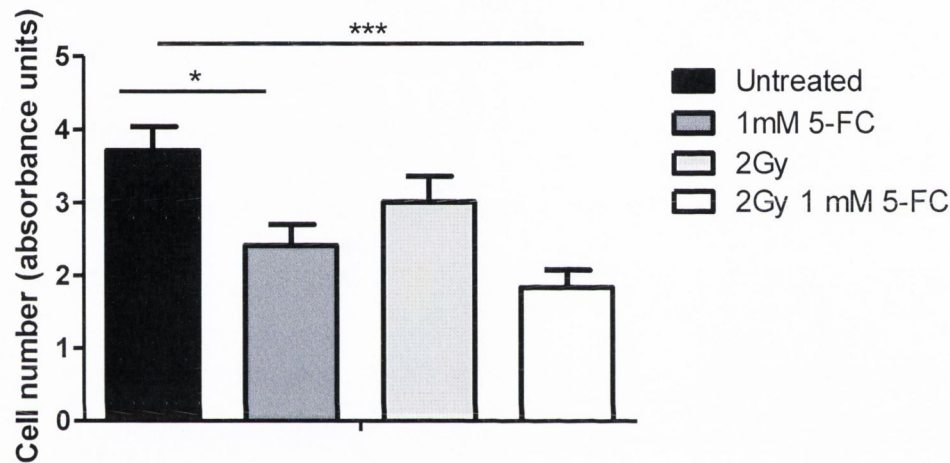


Figure 5.17: Absorbance following MTT assay for pPPE-CD transfected 22Rv1 cells supplemented with DHT and treated with 1mM 5-FC with and without radiation. Values represent the mean of at least three replicates $\pm$ standard error of the mean. ***, $p < 0.001$; *, $p < 0.05$.

5.2.4 Clonogenics assay testing pPPE-CD/5-FC with radiation treatment

Following on from the MTT assays, clonogenic assays were performed to further assess the influence of radiation on pPPE-CD/5-FC activity. For pPPE-CD transfected cells grown in air (21% oxygen) with 2 Gy treatment, 5-FC resulted in a significant decrease in colony forming ability ($p < 0.01$) that was not observed for untransfected cells (Figure 5.18). When cells were grown in hypoxia (0.5% oxygen), treatment with 2Gy and DHT resulted in a significant difference between 5-FC treated and untreated samples ($p < 0.05$, Figure 5.19).

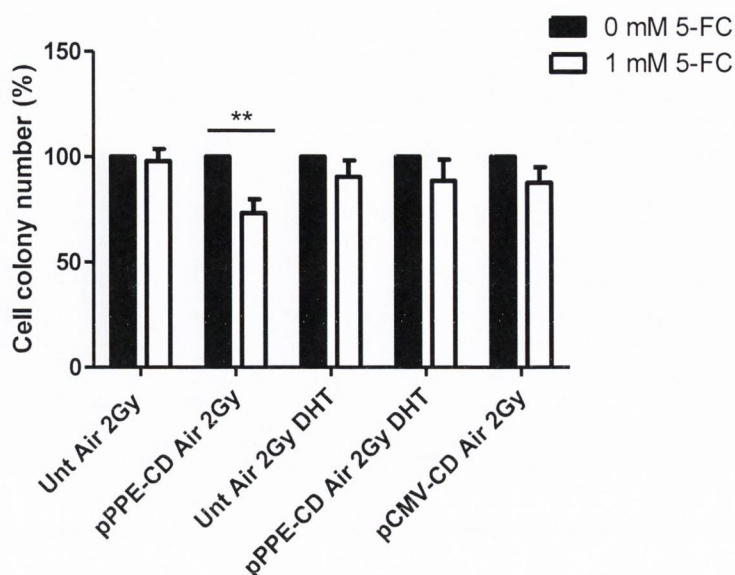


Figure 5.18: Cell colony number following clonogenic assays of 22Rv1 grown in air with 2Gy radiation, with or without DHT and treated with 5-FC. Unt, untransfected; **, $p < 0.01$. Values represent the mean of three replicates $\pm$ standard error of the mean.

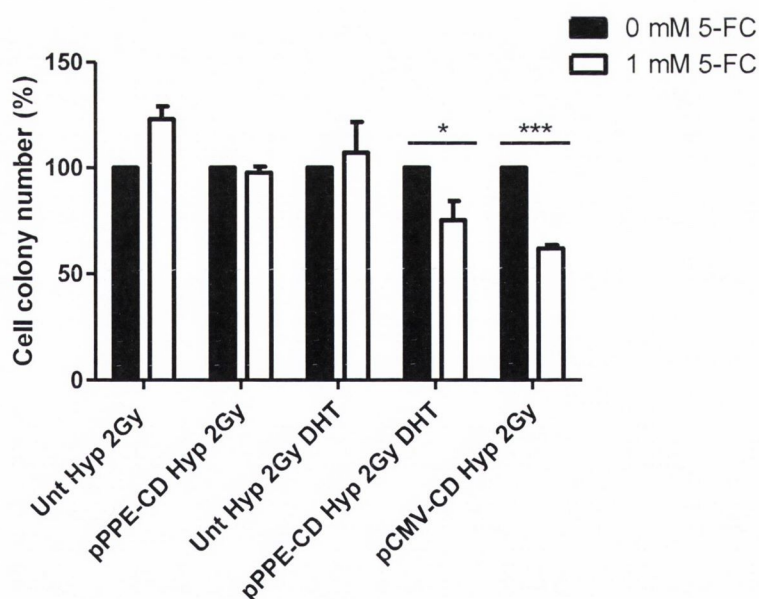


Figure 5.19: Cell colony number following clonogenic assays of 22Rv1 grown in hypoxia with 2Gy radiation, with or without DHT and treated with 5-FC. Unt, untransfected; *, $p < 0.05$; ***, $p < 0.001$. Values represent the mean of three replicates $\pm$ standard error of the mean.

5.2.5 AR expression in response to radiation

To examine the mechanisms controlling the increased CD expression in pPPE-CD-transfected cells treated with radiation, Western blotting of androgen receptor levels was performed on unirradiated/irradiated cell samples grown in air with and without DHT (1000 nM) (Figure 5.20 and Figure 5.21). In both LNCaP and 22Rv1 cell samples, irradiation resulted in higher levels of AR expression. In 22Rv1 cells, the highest levels of both the full length and truncated AR were observed for samples treated with both DHT and radiation. As CD expression is controlled by the PSA promoter and enhancer which have multiple androgen response elements, this may explain the increase in CD levels for irradiated cells.

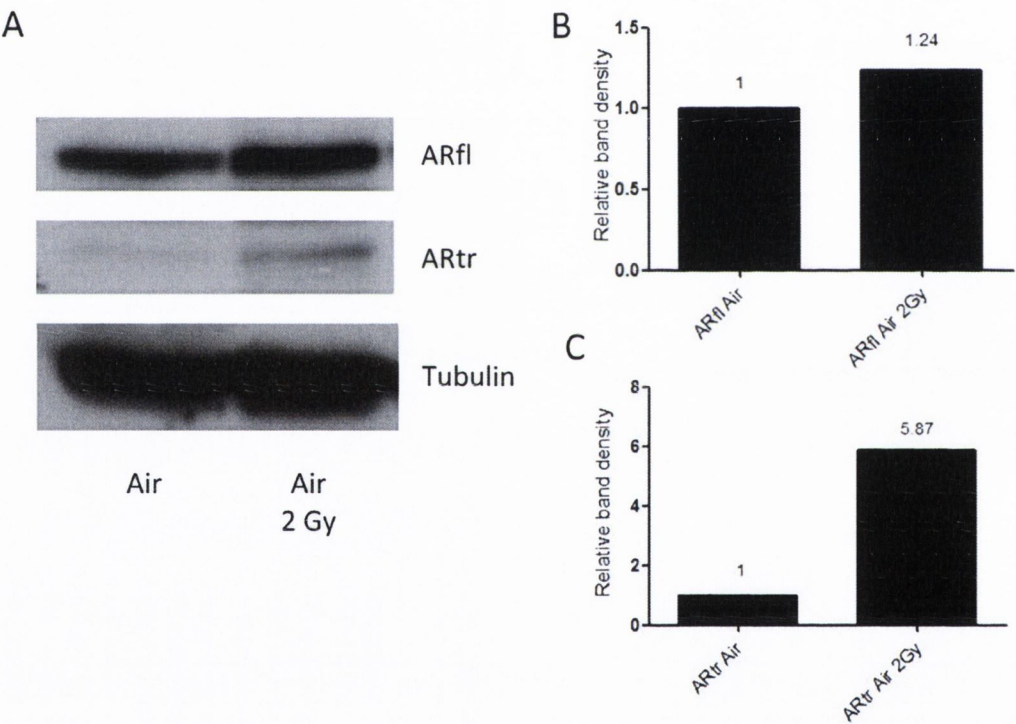


Figure 5.20: A) Androgen receptor protein expression in LNCaP with and without radiation treatment. Tubulin levels were used as a loading control. Representative blot, N=2. 2 Gy, 2 Gy radiation; ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

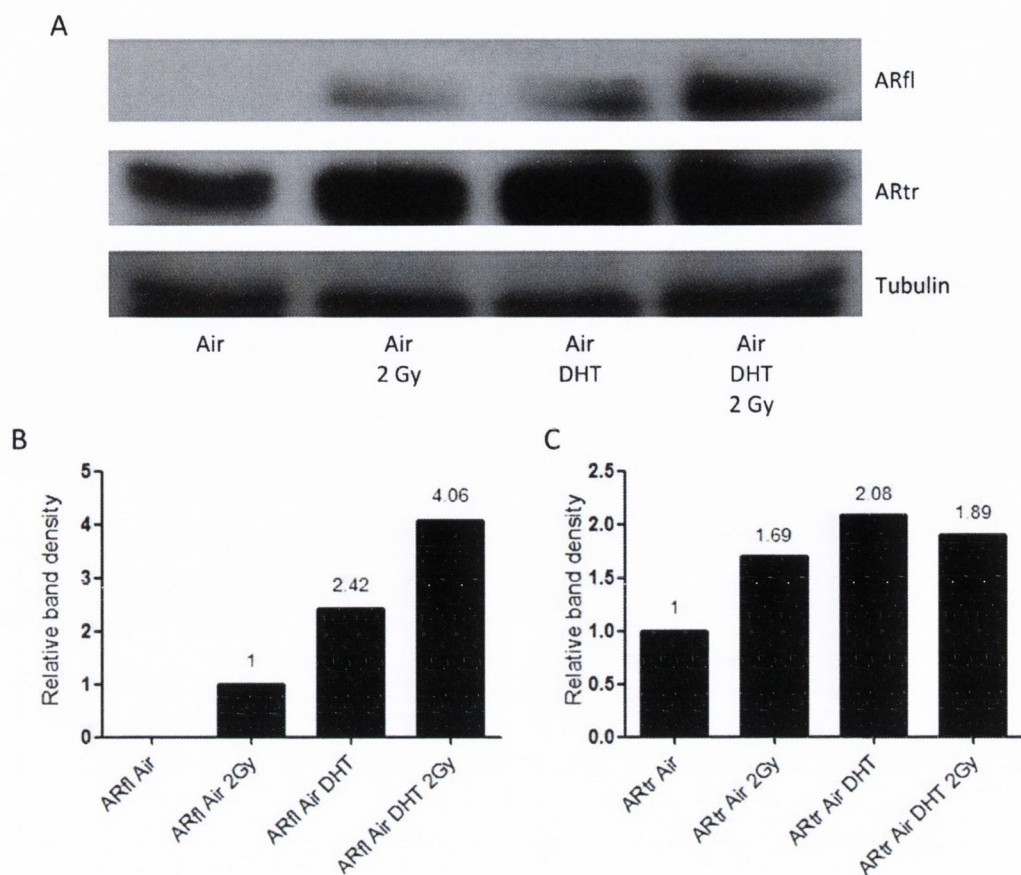


Figure 5.21: A) Androgen receptor protein expression in 22Rv1 cells exposed to radiation and DHT treatment. Tubulin levels were used as a loading control. Representative blot, N=2. 2 Gy, 2 Gy radiation; DHT, 100 nM DHT; ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

The effect of radiation treatment on AR expression was further tested by looking at the gene expression levels using qRT-PCR. The pattern in AR expression was different for LNCaP and 22Rv1. In LNCaP cells, hypoxia (0.5% oxygen) alone was sufficient to significantly increase AR expression levels compared to the aerobic samples (Figure 5.22). Irradiation of the aerobic samples with 2 Gy, 6 Gy or 8 Gy all significantly increased AR levels and this was to a greater extent than hypoxia treatment. A combination of hypoxia and radiation treatments did not result in any further increase in AR expression compared to radiation alone.

In 22Rv1 cells, there was a trend towards higher AR expression with increasing doses of radiation (Figure 5.23). These levels did not reach significance in air (21% oxygen) but when radiation was combined with hypoxia (0.5% oxygen) there was a significant increase in AR levels for 2 Gy and 8 Gy compared to samples from cells grown in air alone. This difference between aerobic and hypoxic AR levels did not reach significance for the unirradiated samples (0 Gy).

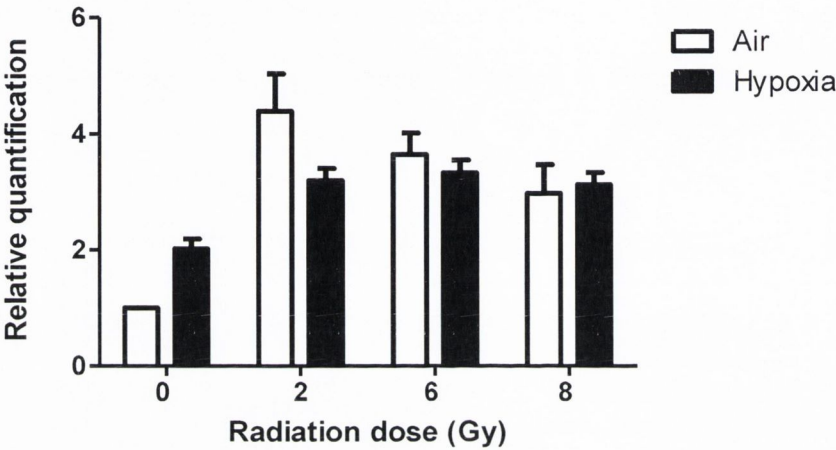


Figure 5.22: Relative quantification of *AR* mRNA in aerobic and hypoxic LNCaP samples treated with various doses of radiation. Values represent the mean of three replicates $\pm$ standard error of the mean.

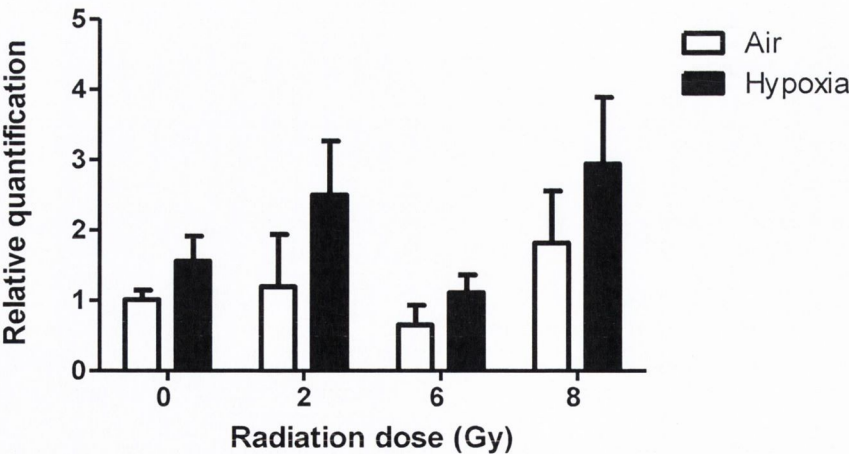


Figure 5.23: Relative quantification of *AR* mRNA in aerobic and hypoxic 22Rv1 samples treated with various doses of radiation. Values represent the mean of three replicates $\pm$ standard error of the mean.

5.2.6 *NF-κB expression in response to radiation*

Levels of the NF-κB1 protein subunits p50 and p105 were examined in 22Rv1 cells grown in air (21% oxygen) and hypoxia (0.5%) with DHT and/or radiation treatment by Western blotting. This was an attempt to further elucidate the mechanisms controlling the upregulation of AR and CD expression in pPPE-CD transfected cells in response to radiation. Other research groups have identified NF-κB binding sites in the genes expressing AR and PSA and NF-κB has been shown to be radiation-responsive. The results of this research project suggested a slight upregulation of NF-κB expression in response to treatment with 2 Gy radiation (Figure 5.24 and Figure 5.25).

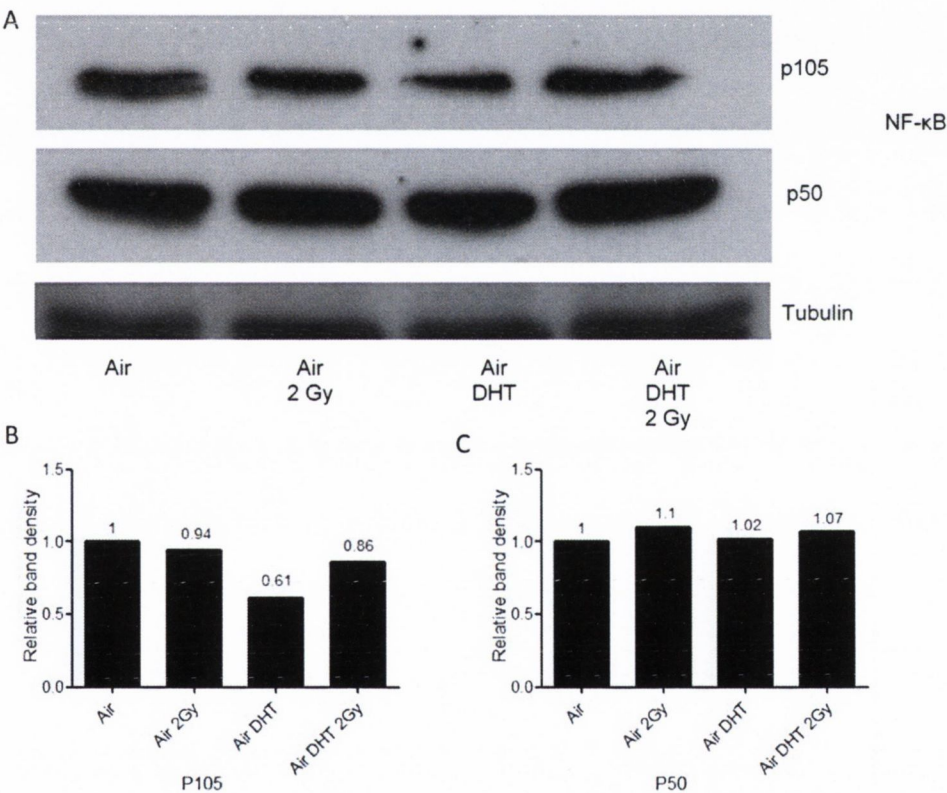


Figure 5.24: A) NF-κB protein expression in 22Rv1 cells exposed to radiation and DHT treatment under aerobic conditions. Tubulin levels were used as a loading control. Representative blot, N=2. DHT, 1000 nM DHT; 2 Gy, 2 Gy radiation. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for P105 (B) and P50 (C).

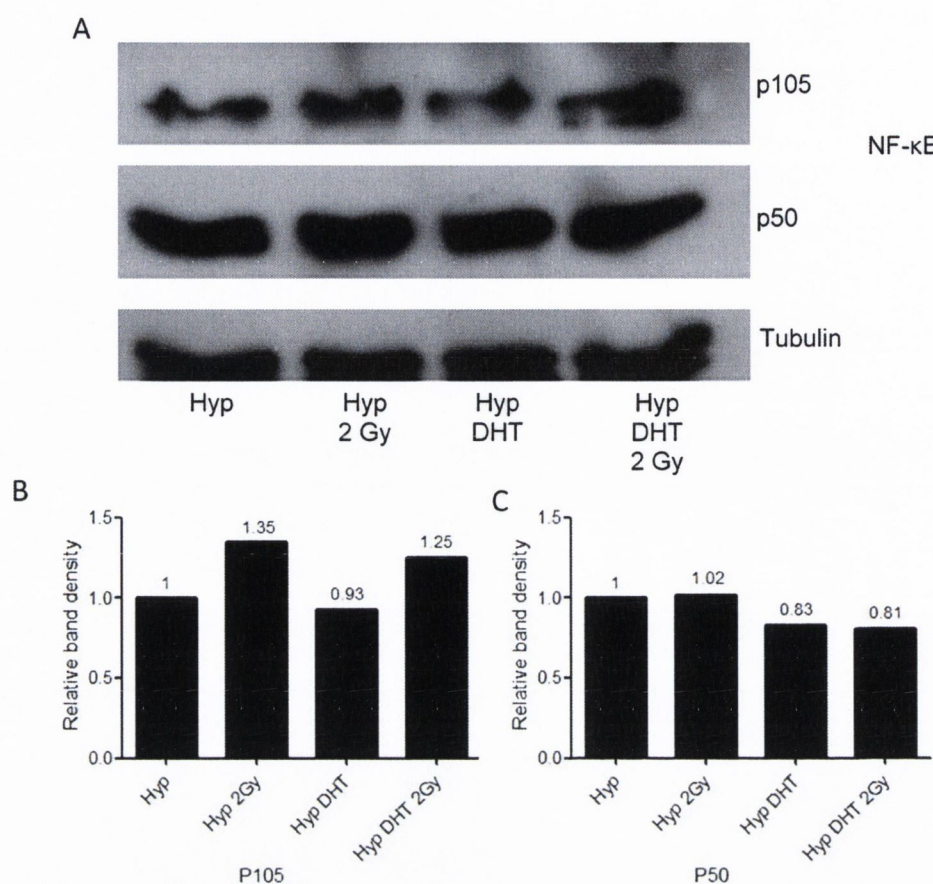


Figure 5.25: A) NF-κB protein expression in 22Rv1 cells exposed to radiation and DHT treatment under hypoxic conditions. Tubulin levels were used as a loading control. Representative blot, N=2. Hyp, Hypoxia; DHT, 1000 nM DHT; 2 Gy, 2 Gy radiation. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for P105 (B) and P50 (C).

5.3 Discussion

One aim of this research was to test pPPE-CD in combination with radiation therapy *in vitro*. The radiosensitization benefits of 5-FU for radiation treatment have previously been established. Before combining the two treatment strategies, experiments were carried out to check if radiation would have any influence on the efficacy of pPPE-CD/5-FC. Initial analysis assessed PSA protein levels in response to 2 Gy radiation given under aerobic (21% oxygen) and hypoxic (0.5%) conditions and there was no significant difference in PSA secretion for samples treated with or without radiation (Figure 5.1 and Figure 5.2). The levels of PSA gene expression were also studied in LNCaP and 22Rv1 cell samples treated with increasing doses of radiation (2 Gy, 6 Gy, 8 Gy) in air and hypoxia. In this case, for LNCaP cells, radiation significantly upregulated PSA gene expression compared to the unirradiated control and the difference was more pronounced when radiation and hypoxia were combined (Figure 5.3). This finding was not replicated in 22Rv1 cells however (Figure 5.4). These results led to the examination of CD protein expression to investigate whether irradiation of the pPPE-CD transfected cells would have any influence on the activity of the vector.

For both LNCaP and 22Rv1-pPPE-CD transfected cells, those given 2Gy of radiation along with DHT treatment seemed to show an increase in CD protein expression compared to DHT alone in 21% oxygen and this appeared to be more pronounced for those grown in 0.5% oxygen. This was a promising result as it suggested there may be a synergistic relationship between the two treatment strategies, with radiation improving the efficacy of pPPE-CD/5-FC as well as the potential increase in radiosensitivity resulting from 5-FU. Subsequent analysis was carried out by measuring the change in cell number using MTT assays in 5-FC treated cells grown under various conditions. In a similar manner to the MTT results in Chapter 4, in which 0.5% oxygen enhanced cell kill, the results were more pronounced for 22Rv1 than LNCaP cells. In LNCaP cells the decrease in cell numbers for pPPE-CD transfected cells did not reach significance under any of the treatment conditions (2 Gy radiation given under aerobic or hypoxic conditions with or without DHT). In 22Rv1 cells, 2 Gy radiation alone was not sufficient to significantly reduce cell number in pPPE-CD-22Rv1 compared to untransfected cells (that is, 2 Gy radiation alone did not activate CD expression which would

result in 5-FC to 5-FU conversion and subsequent cell kill). When 2Gy radiation was combined with DHT treatment in air, the decrease in cell number for pPPE-CD transfectants treated with 1 mM 5-FC was significant compared to untransfected cells ($p < 0.01$). Importantly, this was a greater reduction in cell number than was seen for DHT treatment in air without 2Gy radiation ($p < 0.05$) in Chapter 3, indicating that the upregulation of CD expression observed for radiation treatment translates into higher levels of cells kill and improves the efficacy of the pPPE-CD/5-FC GDEPT system.

These findings were further tested using clonogenic assays. 22Rv1 cells were transfected with pPPE-CD or pCMV-CD and untransfected cells were used as controls. The cells were grown under aerobic (21% oxygen) or hypoxic (0.5%) conditions with or without DHT and treated with 2 Gy radiation before 5-FC treatment. Growth of pPPE-CD cells in air with radiation treatment was sufficient to result in a significant decrease in colony forming ability of the 5-FC treated cells compared to those without 5-FC. Previous results, in Chapter 4, had shown treatment with 5-FC after growth in air alone did not affect colony forming ability so the addition of 2 Gy radiation treatment sufficiently activates pPPE-CD to result in significant toxicity. This replicates the results previously obtained by MTT assay that show 2Gy radiation treatment improves the efficacy of pPPE-CD/5-FC treatment. Under hypoxic conditions, pPPE-CD cells grown with DHT and given 2 Gy radiation treatment showed significantly lower colony numbers for the 5-FC treated samples compared to untreated. This was not the case for untransfected cells given the same treatment. Previously, in Chapter 4, there was no significant difference between hypoxia and DHT grown pPPE-CD transfectants with and without 5-FC.

The results of the Western blots and subsequent MTT and clonogenic assays strongly suggest that radiation, in combination with DHT and/or hypoxic treatments, can further upregulate activity of pPPE-CD and demonstrates the benefits of a combined treatment strategy involving both pPPE-CD/5-FC and radiation treatment.

As was the case with growth in 0.5% oxygen, PSA expression levels in 22Rv1 in response to radiation did not reflect the subsequent CD expression levels in these

cells. PSA gene expression levels were significantly higher for irradiated LNCaP samples compared to unirradiated samples but this was not the case for 22Rv1. However, upregulation of CD expression was observed in both cell lines when pPPE-CD transfected cells were treated with 2 Gy radiation. Furthermore, the more dramatic decrease in cell survival with pPPE-CD/5-FC treatment was observed for 22Rv1 cells. This is further evidence to suggest that there are differences in the sequence of the PSA promoter/enhancer in the vector compared to 22Rv1, resulting in differences in their regulation and expression levels of CD and PSA.

Analysis of protein samples was carried out to try to understand the mechanism controlling the increase in CD expression in irradiated cells as well as the increase in PSA mRNA levels in LNCaP cells in response to radiation treatment. As previously mentioned, the PSA promoter/enhancer contains multiple AREs and is regulated by AR. Therefore, levels of AR protein expression were measured under aerobic conditions in combination with radiation in 22Rv1 and LNCaP. Western blotting showed that treatment with a 2 Gy dose of radiation resulted in an increase in AR levels and the highest levels were for those samples treated with DHT and radiation compared to DHT or radiation alone. qRT-PCR analysis of AR gene expression was carried out to further examine the response to radiation. Cells were treated with increasing doses (2 Gy, 6 Gy, 8 Gy) under aerobic (21% oxygen) and hypoxic (0.5%) conditions and compared to unirradiated cells. The results differed for LNCaP and 22Rv1 cells. In LNCaP, both hypoxia and radiation treatment increase AR expression compared to aerobic samples but a combination of the two did not increase levels any further. In 22Rv1 cells, neither hypoxia nor radiation was sufficient to significantly increase AR expression although there was a trend towards higher levels. The relative quantity reached significance when hypoxia and radiation were combined, compared to aerobic unirradiated samples (Figure 5.23). The levels of AR gene expression in response to radiation correlate with the PSA gene expression levels previously discussed. In LNCaP cells, radiation and hypoxia treatments alone are sufficient to upregulate AR and PSA. In 22Rv1, radiation did not significantly increase levels of AR or PSA.

One possible explanation for the upregulation of AR and PSA gene expression in response to irradiation could be the activation of the transcription factor NF- κ B.

This is a family of proteins p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100, that share a REL homology domain (RHD) and bind, as homo- and heterodimers, to a common sequence in the promoter regions of a multitude of genes.²²² NF- κ B proteins are sequestered in the cytoplasm through the binding of inhibitor proteins, I κ B α and I κ B β . NF- κ B is activated by a host of stimulatory factors, including cytokines, free radicals, stress and, importantly in this context, radiation. This results in the phosphorylation and subsequent proteasomal degradation of the inhibitor proteins and the transport of NF- κ B into the nucleus.²²² Over 200 genes are known to be regulated by NF- κ B. Research has demonstrated that both AR and PSA contain NF- κ B binding sites, although the mechanism of activation is complex and still to be fully elucidated.

Overexpression of the NF- κ B protein p65 has been shown to be sufficient to activate the PSA promoter/enhancer in LNCaP cells.²²⁰ Treatment of the cells with TNF- α or PMA, known NF- κ B activators, stimulates PSA protein expression, with NF- κ B binding directly to four regions in the PSA core enhancer. Zhang *et al.* examined the role of the p65/p50 heterodimer in AR regulation. They transfected LNCaP cells with a p65 expressing vector and found p65 was sufficient to activate the AR promoter in a luciferase reporter construct as well as endogenous AR.²²¹ Increased transactivation with p65 was enhanced with increasing doses of the synthetic androgen R1881. Eight putative NF- κ B binding sites upstream of the AR transcription start site were identified and specific binding of p50 to these sites was demonstrated using ChIP experiments. The drug parthenolide, an NF- κ B inhibitor, decreased AR expression, PSA secretion and cell proliferation.

A further study has shown that in LNCaP cells, transfection with a p52-expressing vector results in expression of PSA protein and mRNA in dose dependent manner and independent of androgen.²²³ ChIP assays have demonstrated that p52 binds to ARE III in the distal PSA enhancer but not ARE I or ARE II. The recruitment of p52 to ARE III and subsequent PSA upregulation is AR dependent. p52 acts to increase recruitment of the AR co-activator p300 to the ARE and also increase transport of AR into the nucleus through a physical interaction with the AR NH₂-terminal domain. Furthermore, the influence of p52 on gene expression is not restricted to PSA as it was shown to also activate NKX3.1.

As a preliminary experiment in the current study, Western blotting for the NF- κ B protein p50/p105 in 22Rv1 cell samples grown in 21% and 0.5% oxygen and treated with 2 Gy radiation was carried out and indicated a slight increase in NF- κ B expression with radiation. However, NF- κ B is mainly regulated post-translationally, so future experiments should involve extraction of nuclear and cytoplasmic protein to determine whether radiation treatment has increased nuclear levels of NF- κ B alongside increased AR expression. If this is the case, the cells should be transfected with the commercially available expression construct for I κ B α M, which is a constitutively active version of the NF- κ B inhibitor or treated with the drug parthenolide, to investigate NF- κ B's possible role in the radiation induced expression of the AR and the upregulation of CD in irradiated pPPE-CD transfected cells.

It has been established that NF- κ B is associated with radioresistance²²⁴ and much work has been carried out to develop NF- κ B inhibitors as a means of increasing the efficacy of radiation treatment.²²² It is possible that the upregulation of AR by NF- κ B may contribute to this radioresistance. Regardless of the upstream mechanisms controlling it, if the observed increase in AR protein and gene expression following irradiation occurs *in vivo* it has implications for radiation treatment and is worthy of further study to establish the upstream signals controlling it.

This research has highlighted the potential benefits of combining radiation therapy with this GDEPT system. Not only is 5-FU a known radiosensitizer but pPPE-CD is activated under hypoxic conditions, which could target those cells resistant to radiation treatment and, in a synergistic manner, radiation could improve the efficacy of pPPE-CD by increasing CD expression levels and be used to further control expression of the vector.

Chapter 6

Creation and characterisation of androgen
receptor-overexpressing 22Rv1

Chapter 6 Creation and characterisation of androgen receptor-overexpressing 22Rv1

6.1 Introduction

Prostate cancer development and progression is characterised by increasing levels of PSA expression and this is used as a biomarker for detection of the disease.¹⁹ 22Rv1 is the best established model of primary prostate cancer but PSA levels expressed by the cells following androgen treatment are relatively low. PSA expression is controlled by the transcription factor AR.²² This chapter concerns the stable transfection of the cell line 22Rv1, with the aim of overexpressing AR. It was hoped that overexpression of AR would produce clinically relevant PSA levels and better reflect the conditions in malignant cells *in vivo*. Since pPPE-CD activity is controlled by the PSA promoter/enhancer, investigating the activity of the vector in this cell line may give a more accurate assessment of its behaviour *in vivo*.

The 22Rv1 cell line was created in 1999, derived from the primary human prostate cancer xenograft CWR22R.²¹⁸ 22Rv1 cells form tumours in nude mice which secrete PSA into their circulation. It was demonstrated by Western blotting that 22Rv1 express AR, which makes it one of the few prostate cancer cell lines that does.^{218, 225} 22Rv1 cells are androgen responsive and increased growth has been demonstrated with androgen treatment but they are not androgen-dependent and can grow in its absence.²²⁵ The AR in 22Rv1 cells has a point mutation, H874Y. Attardi *et al.* demonstrated that there is increased affinity of androgens for AR binding in 22Rv1 cells and decreased specificity of the mutant AR compared to the wild type.²²⁵

As mentioned in section 1.3, 22Rv1 expresses both a full length (flAR) and truncated version of AR (trAR). Research into the mechanisms controlling the production of the shorter AR alongside the full-length version has elucidated several possible pathways, which may work in combination.²²⁶ Alternative splicing of AR generates a number of AR variants that lack the ligand binding domain and of these, AR3 appears to be the main truncated isoform found in prostate cancer cell lines as well as human tissue.^{227, 228} It is found in all AR-expressing cell lines, but at much

higher levels in the androgen-independent ones.²²⁷ AR3 is overexpressed in LNCaP and has been shown to be unaffected by DHT, bicalutamide or AR.²²⁷

Another possible mechanism for production of the shortened AR is cleavage of the carboxy-terminal domain by calpains, which are calcium-dependent proteinases.²²⁹ In LNCaP cells, addition of calcium chloride or the calcium ionophore ionomycin, to activate calpains, results in formation of trAR, which is inhibited by treating the cells with a calpain inhibitor.²²⁹ Treatment of 22Rv1 cells with calpain 2 results in detection of the trAR only, as all the flAR is converted to the shortened form. As previously mentioned, 22Rv1 cells have an exon 3 duplication in AR. Chen *et al.* used AR transfected PC3 cells to demonstrate that this mutation is more sensitive to calpain proteolysis than the wild type AR.²³⁰

The two mechanisms of generation of trAR (alternative splicing and calpain cleavage of AR) are not mutually exclusive. Multiple truncated forms of AR have been identified which may not be identical but share the absence of a ligand binding domain. Chen *et al.* point out that inhibition of calpain 2 does not completely eliminate trAR expression, and these remaining isoforms may be a result of alternative splicing.²³⁰ An analogous process occurs in the case of cyclin E, which can be found as a low molecular weight form in cancer cells and is produced as a result of both alternative splicing²³¹ and calpain proteolysis.²³²

The AR expressing vector pcDNA3.1-AR, used in this research chapter, has previously been used by Waltering *et al.* to create two stably transfected LNCaP cell lines, LNCaP-ARmo (2-4 times higher AR protein expression) and LNCaP-ARhi (4-6 times).²³³ The proliferation rate in these cells was higher than the parent cell line both in the presence and absence of DHT. Genome-wide expression profiling of the cell lines, as well as VCaP (Vertebral-Cancer of the Prostate), was carried out following treatment of the cells with various concentrations of DHT. VCaP is derived from a hormone-refractory metastatic tumour and contains an amplified AR gene locus.²³⁴ The profiling showed clustering of LNCaP-ARhi with VCaP, despite them being different cell lines with different genetic backgrounds. Waltering *et al.* suggest this contributes to the evidence of the strong influence of AR on genome-wide gene expression. AR overexpression resulted in a greater number of genes up and down-regulated in response to androgen-stimulation with the highest number in

VCaP. In response to DHT, PSA mRNA levels were increased in LNCaP-ARmo and LNCaP-ARhi compared to LNCaP, with a maximum difference of 3-4 fold. The stably transfected cells were sensitized to lower concentrations of androgen, showing the same level of gene expression, on average, with a 10-fold lower level of DHT.

Despite the encouraging results assessing the activity of pPPE-CD in 22Rv1 in Chapters 4 and 5, it was hypothesised that the system would perform better in a model with increased activity of the PSA promoter and that the full potential of pPPE-CD is not being realised in 22Rv1 cells. Given that previous research creating AR-overexpressing LNCaP cells has been shown to result in the increased activity of AR-responsive genes, it was thought that overexpression of AR in 22Rv1 could yield higher activity of the PSA promoter, thus serving as a better model of *in vivo* conditions in the assessment of the pPPE-CD/5FC GDEPT system.

In this chapter, as well as *PSA*, the levels of a second AR-responsive gene, *FKBP51*, are also compared between the parent cell line and the 22Rv1 cells overexpressing AR. FKBP51 is a member of the immunophilins. Its function has not been fully elucidated but is thought to be involved in protein folding as well as the signal transduction of androgens and other hormones as it has a role as a co-chaperone in heat-shock protein complexes.²³⁵ Levels of FKBP51 protein expression have been shown to be significantly higher in prostate tumour compared to normal samples.²³⁶

Because of its importance in prostate cancer progression, AR is often targeted during treatment using androgen deprivation therapy. The AR may be blocked by anti-androgens such as bicalutamide or leutinizing hormone releasing hormone (LHRH) agonists may be used to reduce the amount of androgen.^{95, 96} Progression to CRPC is an inevitability and is strongly associated with AR overexpression, mainly due to transcriptional upregulation with gene amplification in 10-20% of cases.^{237, 238} Therefore, this chapter also compares bicalutamide response between 22Rv1 and the AR overexpressing 22Rv1 to see if there is any difference in toxicity of the drug.

6.1.1 Aims and Objectives

Aim: Create a novel 22Rv1 cell line that stably overexpresses AR.

Aim: Evaluate the effect of AR overexpression on AR-responsive PSA and FKBP51.

Aim: Evaluate whether AR overexpression enhances the activity of pPPE-CD under a variety of treatment conditions.

Aim: Compare bicalutamide response between the parent and stably transfected 22Rv1 cells.

6.2 Results

6.2.1 Restriction digest

A restriction digest was performed to confirm pcDNA3.1-AR had the expected sequence and size. The restriction enzymes HindIII and BglII were used, with uncut DNA included as a control. The samples were run on a 1% gel and as expected, a single band, indicating a linearized plasmid, was obtained when either HindIII or BglII were used (Figure 6.1).

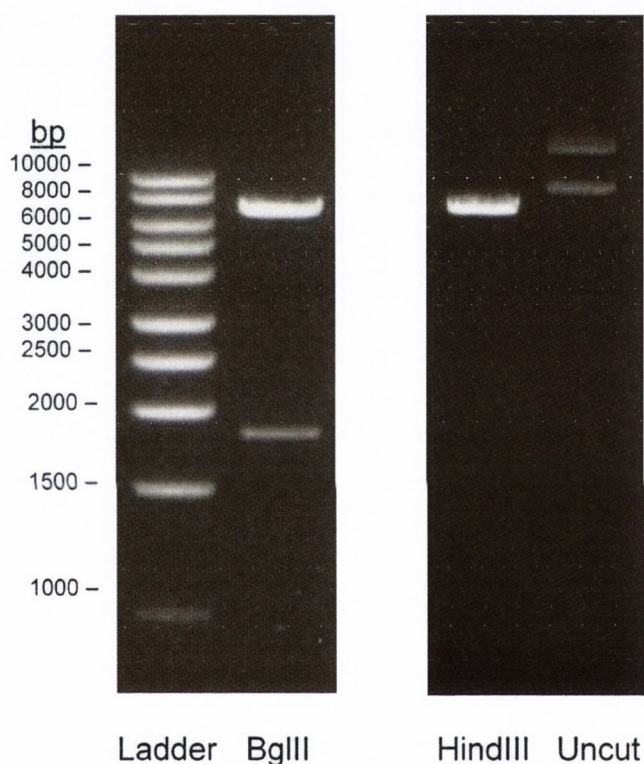


Figure 6.1: pcDNA3.1-AR following digestion with the restriction enzymes BglII and HindIII, as well as uncut plasmid as a control.

6.2.2 Optimisation of Geneticin concentration

The vector pcDNA3.1-AR contains AR as well as the gene for resistance to the antibiotic Geneticin. Before stable transfection of the cells with the vector, the optimal concentration of Geneticin for 22Rv1 cell kill was assessed so that it could be used to distinguish cells that contain pcDNA3.1-AR from those that do not. Following treatment of 22Rv1 cells with a range of concentrations of Geneticin for two weeks, the cells were stained using trypan blue and counted. The level of cell survival for each concentration was calculated as a percentage of the untreated

control cells. 400 $\mu\text{g}/\mu\text{l}$ was selected as the optimal concentration for treatment of the cells following transfection (Figure 6.2). Those cells that had been successfully transfected would be resistant to Geneticin, whereas normal 22Rv1 cells would be killed.

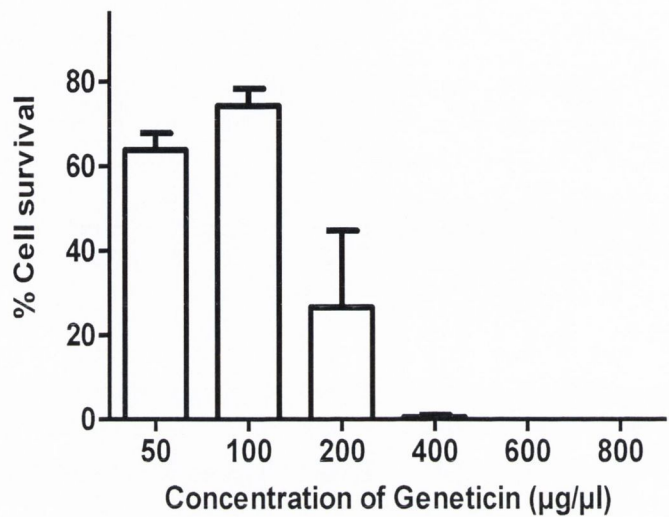


Figure 6.2: Cell survival of 22Rv1 following Geneticin treatment. Values represent the mean $\pm$ standard error of the mean.

6.2.3 AR protein levels

Following transfection of 22Rv1 cells with pcDNA3.1-AR, cell cloning by serial dilution and growth of the transfected cells in Geneticin-containing media, the cells were assessed to determine if the transfection was successful. AR expression levels were higher in the stably transfected cell line 22Rv1-ARhi compared to the parent cell line 22Rv1 when the protein levels were tested using Western blotting (Figure 6.3). The increase is seen for both the full length (114 kDa) and the truncated AR (75-80 kDa). LNCaP expresses high levels of AR and was used as a positive control. The cell lines were also grown with and without DHT (1000nM) prior to protein collection. Upregulation of AR is higher in 22Rv1-ARhi compared to 22Rv1 (Figure 6.4).

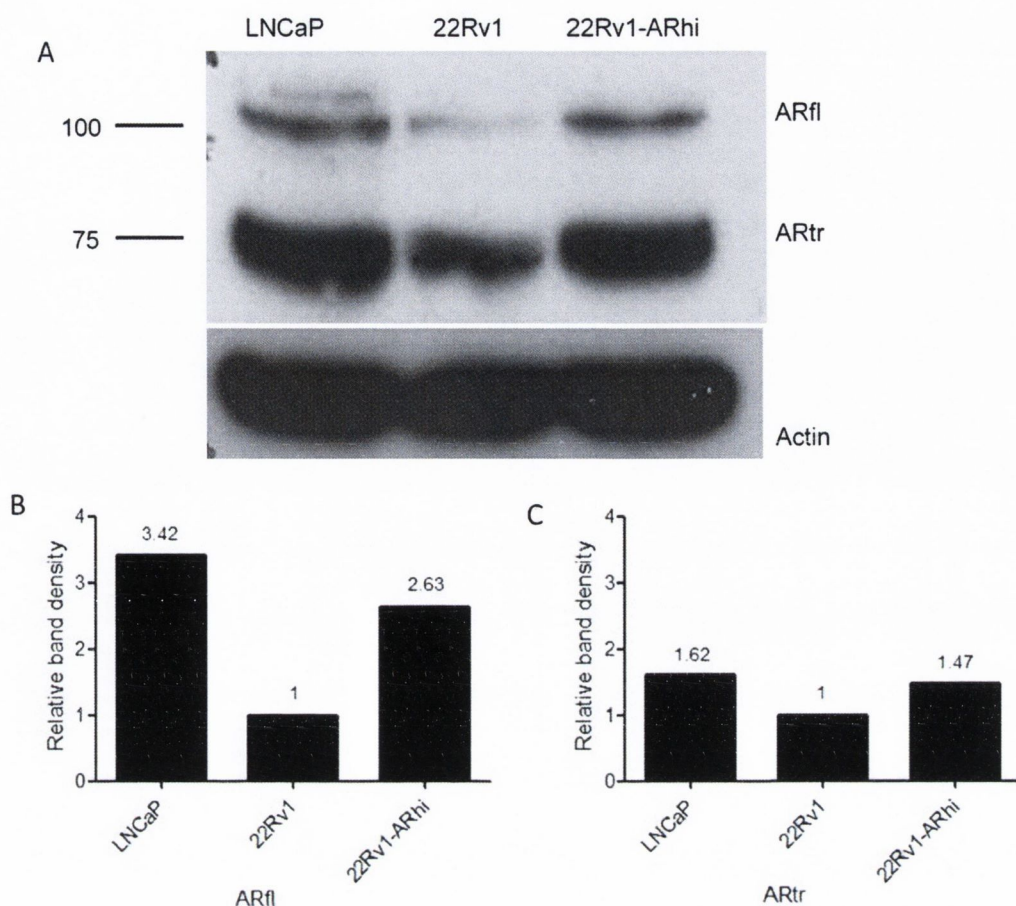


Figure 6.3: A) AR expression levels measured by Western Blotting. Actin levels were used as a loading control. Representative blot, N=2. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

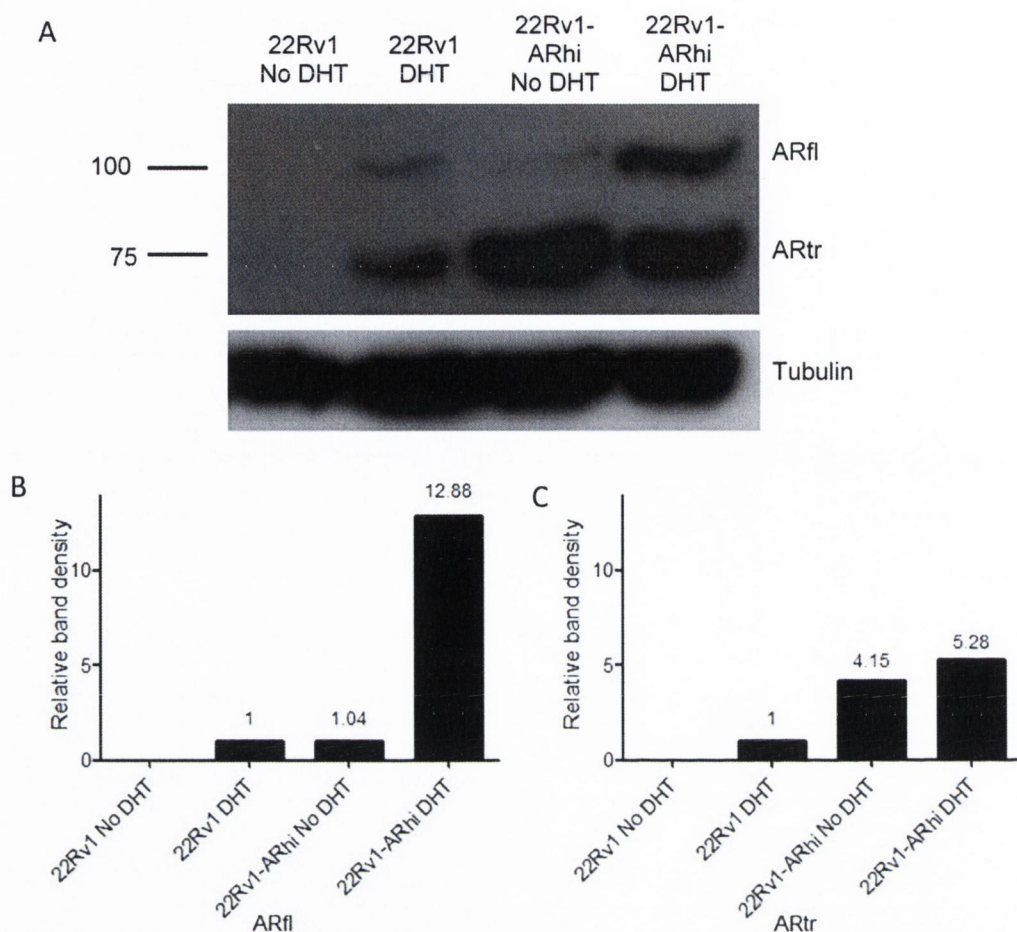


Figure 6.4: A) AR protein expression in response to DHT treatment. Tubulin levels were used as a loading control. Representative blot, N=2. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

6.2.4 Relative quantification of AR gene expression

22Rv1-ARhi was further assessed by measuring the relative quantification of AR expression and comparing it to 22Rv1 and LNCaP (Figure 6.5). A two-tailed unpaired t-test was used to compare the level of expression of the AR gene between 22Rv1 cells and the stably transfected cell line 22Rv1-ARhi. The difference was significant for both the untreated samples and those grown in media with charcoal dextran stripped FBS supplemented with 1000 nM DHT ($p = 0.0171$ and $p = 0.0375$ respectively).

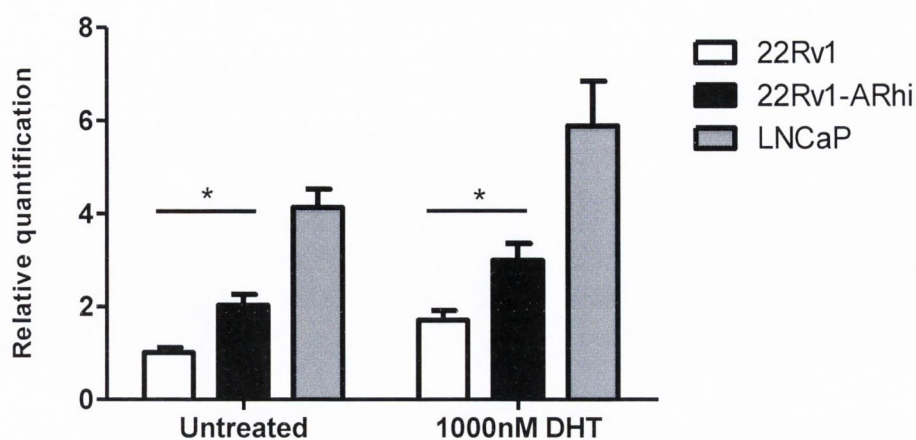


Figure 6.5: Quantification of AR mRNA levels relative to 22Rv1 untreated. Values represent the mean of three replicates $\pm$ standard error of the mean.

6.2.5 PSA protein levels

PSA EIAs were carried out to compare the levels of PSA secretion of 22Rv1-ARhi cells and the parent cell line to determine whether the overexpression of AR resulted in higher levels of PSA protein. A range of DHT concentrations (0.1 – 1000 nM) were tested under both aerobic (21% oxygen) and hypoxic (0.5%) conditions. Media was collected for the EIAs and an MTT assay was carried out on each sample to allow normalisation of the PSA measurements to cell number. The levels of secreted PSA protein were, in fact, lower for 22Rv1-ARhi than 22Rv1, significantly so for some DHT concentrations (Figure 6.6).

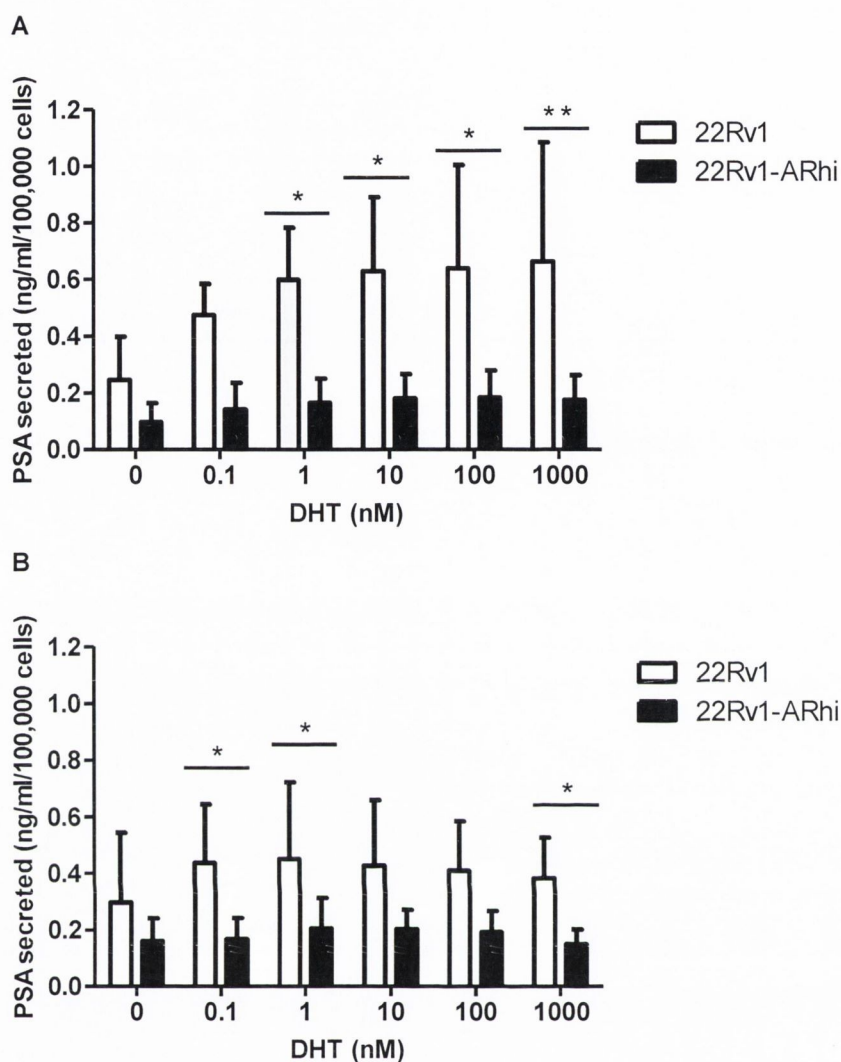


Figure 6.6: PSA protein levels following DHT stimulation in 22Rv1 under aerobic (A) and hypoxic conditions (B) for 72 hours. Values represent the mean of four replicates $\pm$ standard error of the mean.

6.2.6 Relative quantification of PSA gene expression

qRT-PCR analysis was used to calculate the relative level of PSA gene expression in 22Rv1-ARhi compared to normal 22Rv1 when treated with a range of DHT concentrations. The level of PSA for untreated 22Rv1 was used as the calibrator sample (Figure 6.7). As was the case with protein expression, PSA gene expression was lower in stably transfected cell line compared to normal 22Rv1 and the difference was significant for all concentrations of DHT. A similar trend was

observed under conditions of 0.5% oxygen, with lower levels of PSA gene expression for 22Rv1-ARhi (Figure 6.8).

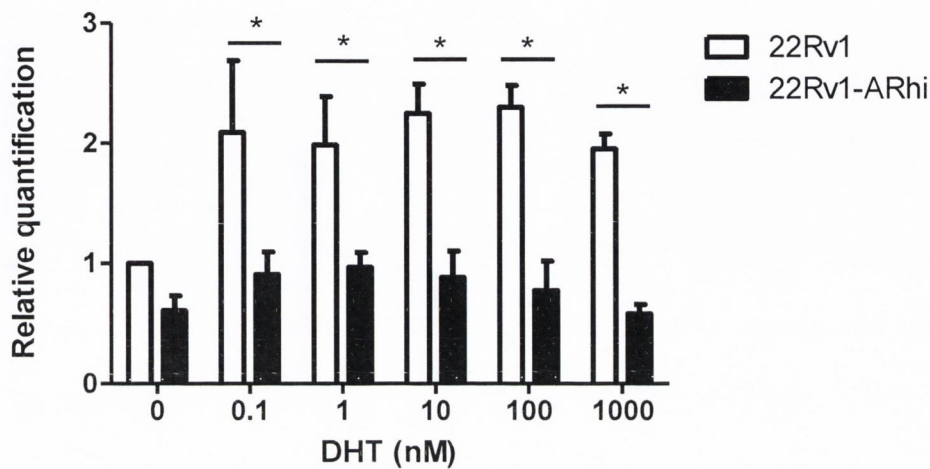


Figure 6.7: Quantification of PSA mRNA levels relative to 22Rv1 untreated under aerobic conditions. Values represent the mean of three replicates $\pm$ standard error of the mean.

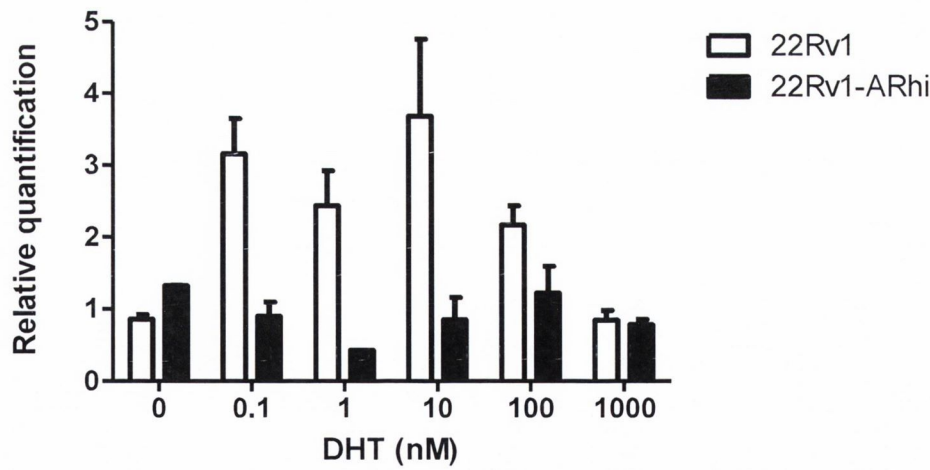


Figure 6.8: Quantification of PSA mRNA levels relative to 22Rv1 untreated under hypoxic conditions. Values represent the mean of three replicates $\pm$ standard error of the mean.

6.2.7 Relative quantification of FKBP51 gene expression

In addition to *PSA*, a second gene, *FKBP51*, was selected as a marker of AR activity. Levels of *FKBP51* gene expression were compared between 22Rv1-ARhi and 22Rv1 cells treated with increasing DHT concentrations in air (21% oxygen) and hypoxia (0.5%) (Figure 6.9). There was no significant difference between the expression levels of the two cell lines but for 0 nM, 1nM and 10 nM DHT there was a trend towards higher expression levels of *FKBP51* for 22Rv1-ARhi under both aerobic and hypoxic conditions. The low level of *PSA* expression in 22Rv1-ARhi was not replicated for *FKBP51* gene expression.

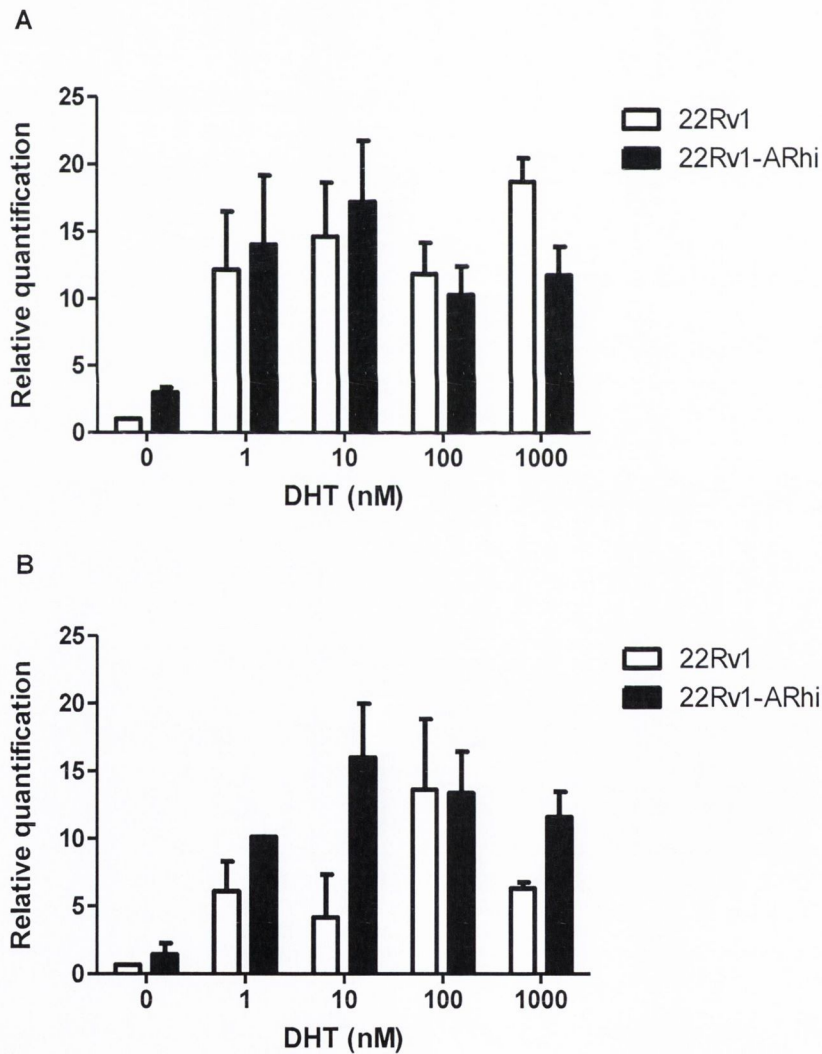


Figure 6.9: Relative quantification of FKBP51 expression in 22Rv1 and 22Rv1-ARhi under aerobic (A) and hypoxic (B) conditions.

6.2.8 Growth rates

The amount of proliferation following DHT treatment was compared between 22Rv1 and 22Rv1-ARhi to determine if AR overexpression results in an increased growth rate. The cells were seeded in CD-FBS media in T75 flasks at a concentration of 0.75×10^6 . Various concentrations of DHT were added and the media/DHT was replaced once during a 7 day incubation period, after which the number of cells were counted using trypan blue. There was no significant difference between the cell numbers for the two cell lines at any of the DHT concentrations (Figure 6.10). There was an increase in cell number with the addition of 10 nM and 100 nM DHT compared to those grown in CD-FBS alone for both cell lines but this only reached significance for 22Rv1-ARhi, 100 nM DHT ($p < 0.05$).

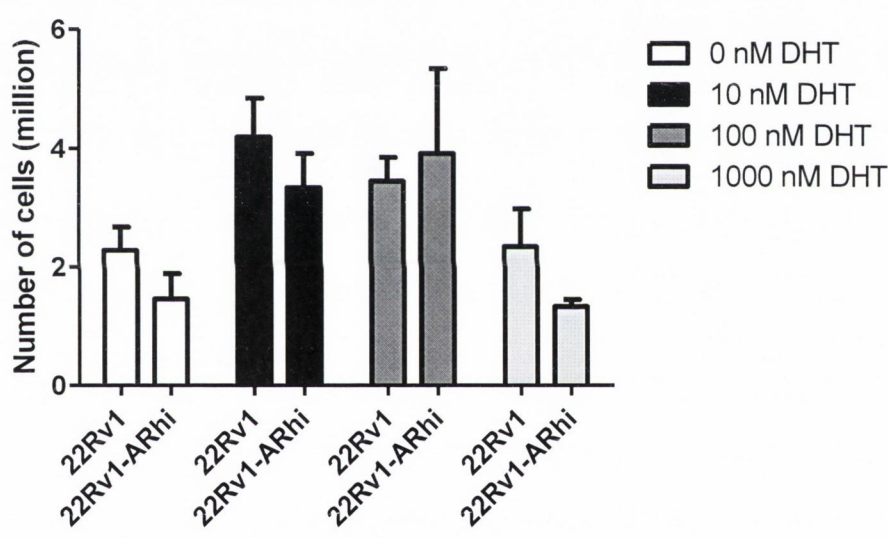


Figure 6.10: Cell count following treatment with various concentrations of DHT for seven days. Values represent the mean of three replicates $\pm$ standard error of the mean.

To further compare the growth rates between 22Rv1-ARhi and the parent cell line, MTT assays were carried out to assess the number of viable cells after growth in CD-FBS media with DHT for 3 days. A larger range of DHT concentrations were used, from 0.1 nM to 1000 nM and the cells were grown in both aerobic (21% oxygen) and hypoxic (0.5%) conditions. There was no significant difference between the cell lines at any of the DHT concentrations for air (Figure 6.11) or hypoxia (Figure 6.12). Comparing aerobic and hypoxic growth rates for each cell

line, the difference was significant at all DHT concentrations for 22Rv1 ($p < 0.001$) but was not significant for 22Rv1-ARhi.

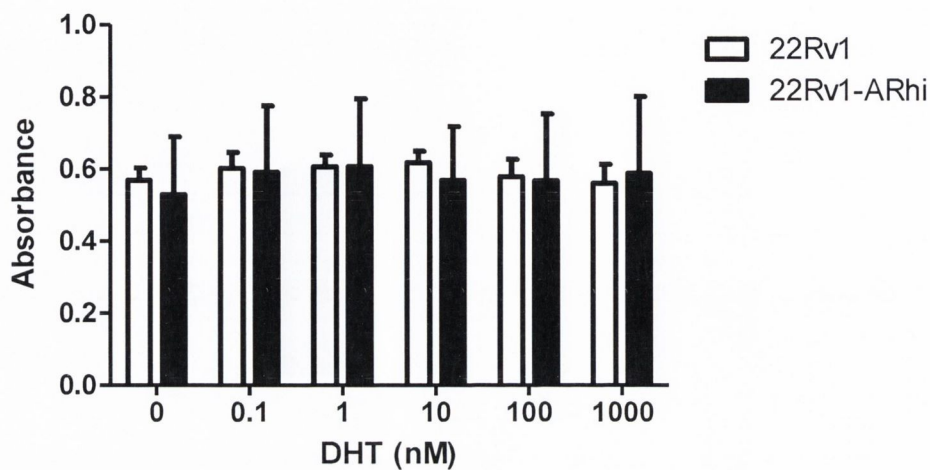


Figure 6.11: Absorbance levels after MTT assay of cells grown for 3 days with various concentrations of DHT under aerobic conditions. Values represent the mean of three replicates $\pm$ standard error of the mean.

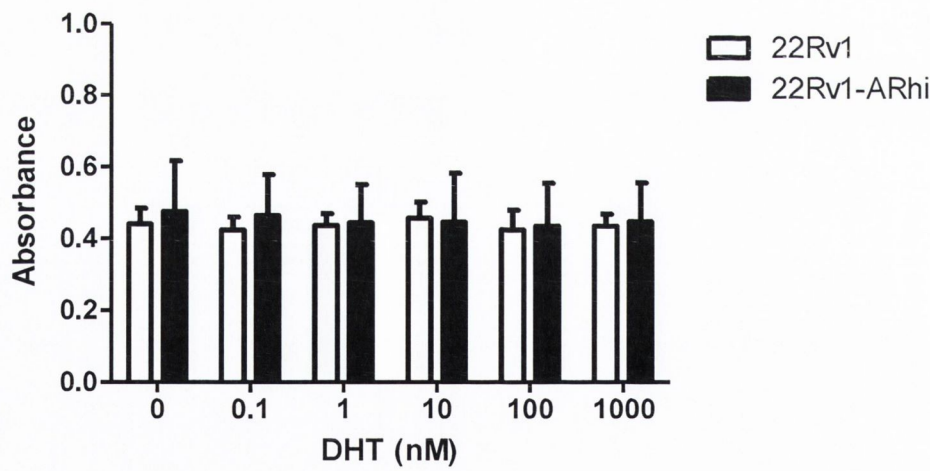


Figure 6.12: Absorbance levels after MTT assay of cells grown for 3 days with various concentrations of DHT under hypoxic conditions. Values represent the mean of three replicates $\pm$ standard error of the mean.

6.2.9 Efficiency of CD expression in pPPE-CD transfected 22Rv1-ARhi

22Rv1 and 22Rv1-ARhi were transfected with pPPE-CD and treated with various combinations of DHT and radiation under aerobic (21% oxygen) and hypoxic (0.5%) conditions. The levels of CD expression were assessed using Western blotting. The same patterns of expression were seen in both cell lines in response to the various treatments. There was an increase in CD protein levels in hypoxic compared to aerobic samples (Figure 6.13) and an upregulation of protein expression when radiation and DHT are combined (Figure 6.14). As may have been expected from the low PSA expression results, CD expression was consistently lower in 22Rv1-ARhi compared to the parent cell line 22Rv1.

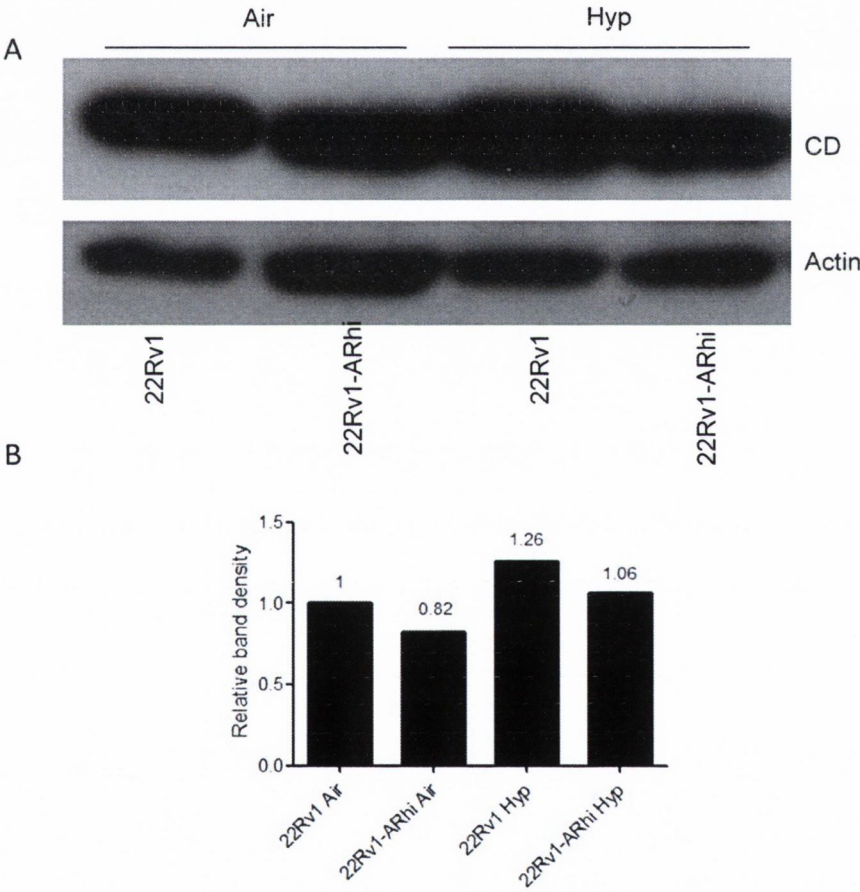


Figure 6.13: A) CD expression in pPPE-CD transfected 22Rv1 and 22Rv1-ARhi cells under aerobic and hypoxic conditions. Actin levels were used as a loading control. Representative blot, N=2. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

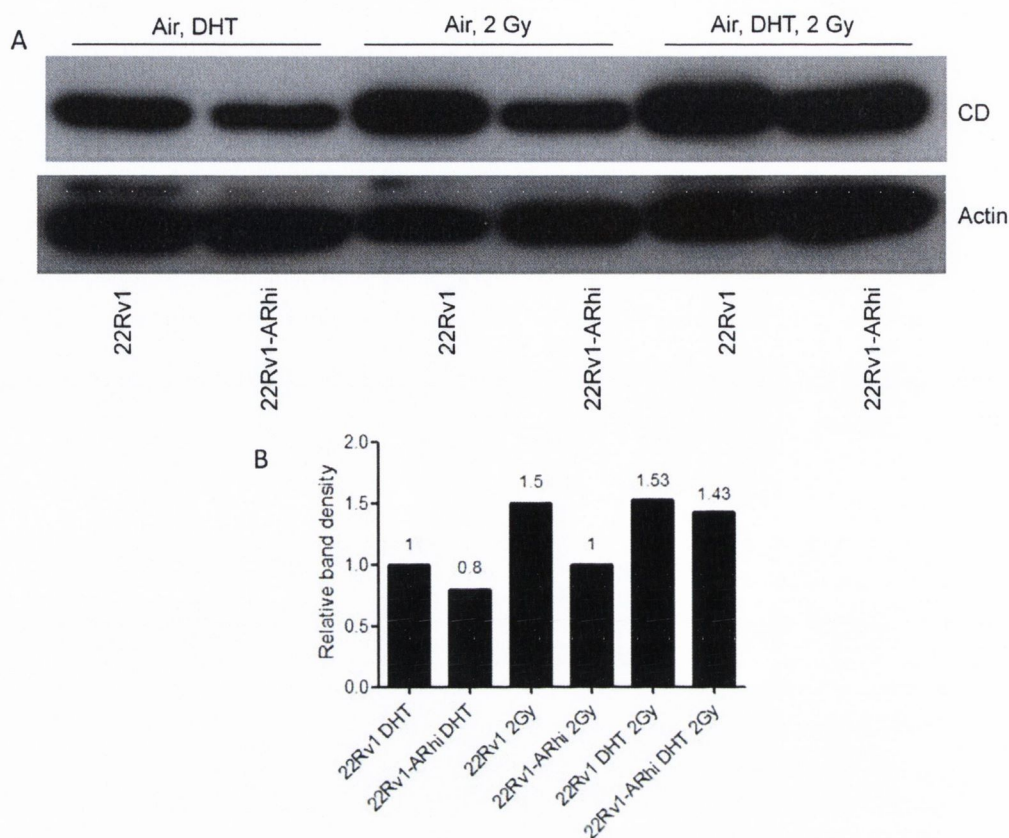


Figure 6.14: A) CD expression in pPPE-CD transfected 22Rv1 and 22Rv1-ARhi cells grown under aerobic conditions with DHT and/or radiation treatment. Actin levels were used as a loading control. Representative blot, N=2. B) Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band.

6.2.10 Response to Bicalutamide treatment

One mechanism suggested for the development of resistance to bicalutamide in prostate tumours is the overexpression of AR and this has been demonstrated *in vitro* in LNCaP cells. The effect of bicalutamide treatment was compared between 22Rv1-ARhi and the parent cell line to assess whether increased AR expression influenced response in this case.

MTT assay

The cells were treated with bicalutamide in DHT supplemented CD-FBS media under aerobic (21% oxygen) and hypoxic (0.5%) conditions and an MTT assay was

used to measure the number of viable cells. The results for each of the bicalutamide concentrations are expressed as a percentage of the number of untreated (DHT only) control cells. There was no significant difference between the number of cells for 22Rv1, 22Rv1-AMC or 22Rv1-ARhi for any of the three bicalutamide concentrations tested (1, 10, 50 μ M) under either aerobic (Figure 6.15) or hypoxic conditions (Figure 6.16). The efficacy of bicalutamide toxicity appears to be reduced under hypoxic conditions. 1 μ M concentration of the drug was sufficient to result in a significant decrease in viable cells compared to those treated with DHT alone for all three cell lines. This was not the case for hypoxic cells, where a significant decrease was only observed at the highest bicalutamide concentration, 50 μ M, and then only for 22Rv1 and 22Rv1-ARhi.

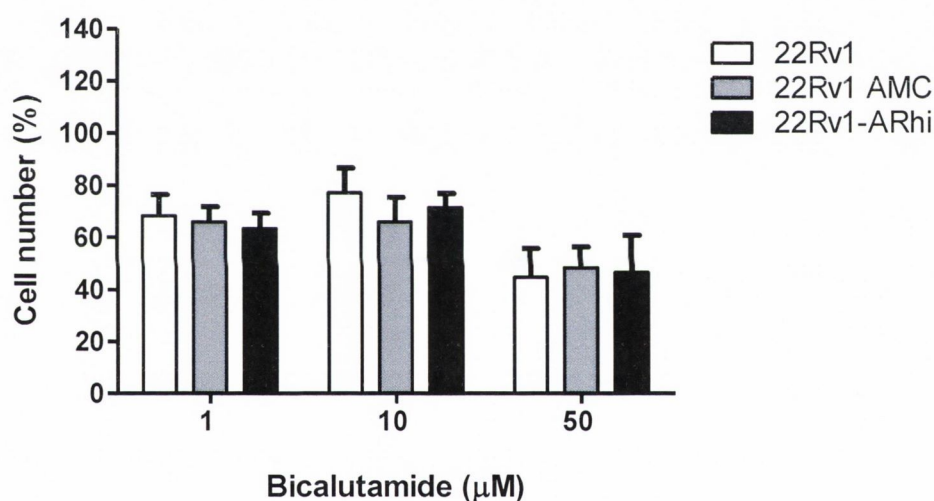


Figure 6.15: Cell number following bicalutamide treatment under aerobic conditions. Values represent the mean of five replicates $\pm$ standard error of the mean.

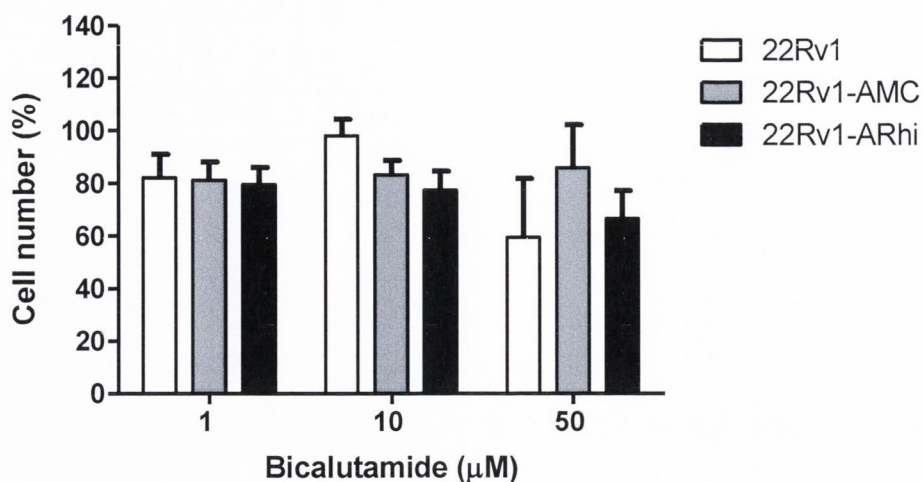


Figure 6.16: Cell number following bicalutamide treatment under hypoxic conditions. Values represent the mean of three replicates $\pm$ standard error of the mean.

Clonogenic assay

To further test the response of 22Rv1-ARhi cells to bicalutamide treatment, clonogenic assays were carried out. The cells were seeded in androgen-depleted media, followed by treatment under aerobic conditions with 10 μ M or 50 μ M bicalutamide in media supplemented with 1000 nM DHT or with ethanol and DMSO as controls for DHT and bicalutamide respectively. The results were calculated as a percentage of the untreated control (DHT only) for each cell line. The results of the clonogenic assays concurred with the previous MTT results, with no significant difference between the response of 22Rv1 and 22Rv1-ARhi cells (**Figure 6.17**).

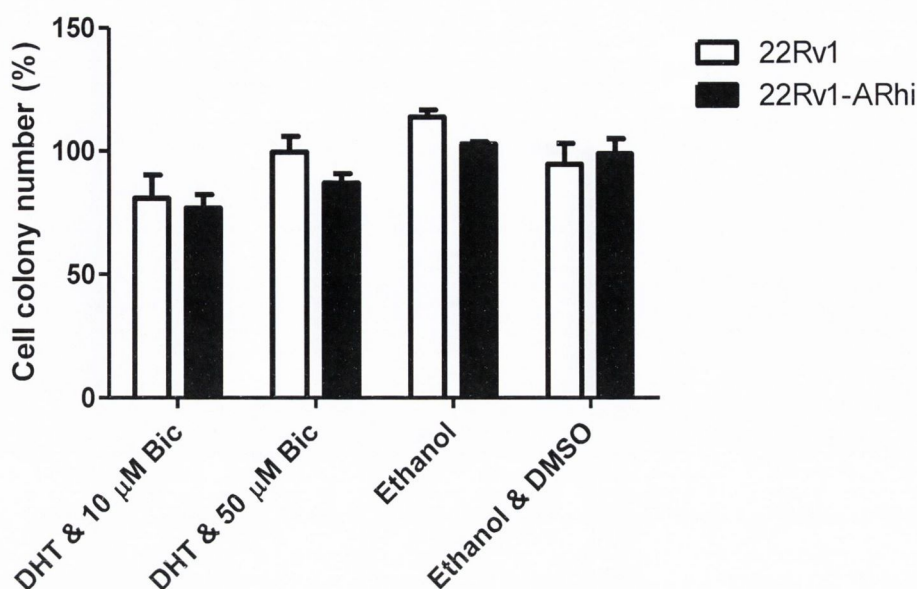


Figure 6.17: Cell colony number following bicalutamide treatment. Values represent the mean $\pm$ standard error of the mean. Bic, bicalutamide.

6.2.11 Cytoplasmic and nuclear protein levels of AR

To try to understand why AR overexpression did not translate to an increase in PSA expression, LNCaP, 22Rv1 and 22Rv1-ARhi cells were treated with a range of DHT concentrations. Nuclear and cytoplasmic protein was then extracted to look at the level of transport of AR to the nucleus following DHT stimulation. For the untreated cells (0 nM DHT), truncated AR expression was detected for 22Rv1-ARhi but not for 22Rv1 (Figure 6.18). When 0.1 nM DHT was used, the AR expression levels were higher for 22Rv1, for both full length and truncated AR (Figure 6.18). At 10 nM DHT, the AR protein levels in the nucleus were very similar for 22Rv1-ARhi and the parent cell line (Figure 6.19). Interestingly, it appears that at the highest DHT concentration of 1000 nM, AR protein levels in the nucleus are significantly reduced in 22Rv1-ARhi cells compared to 22Rv1 (Figure 6.19).

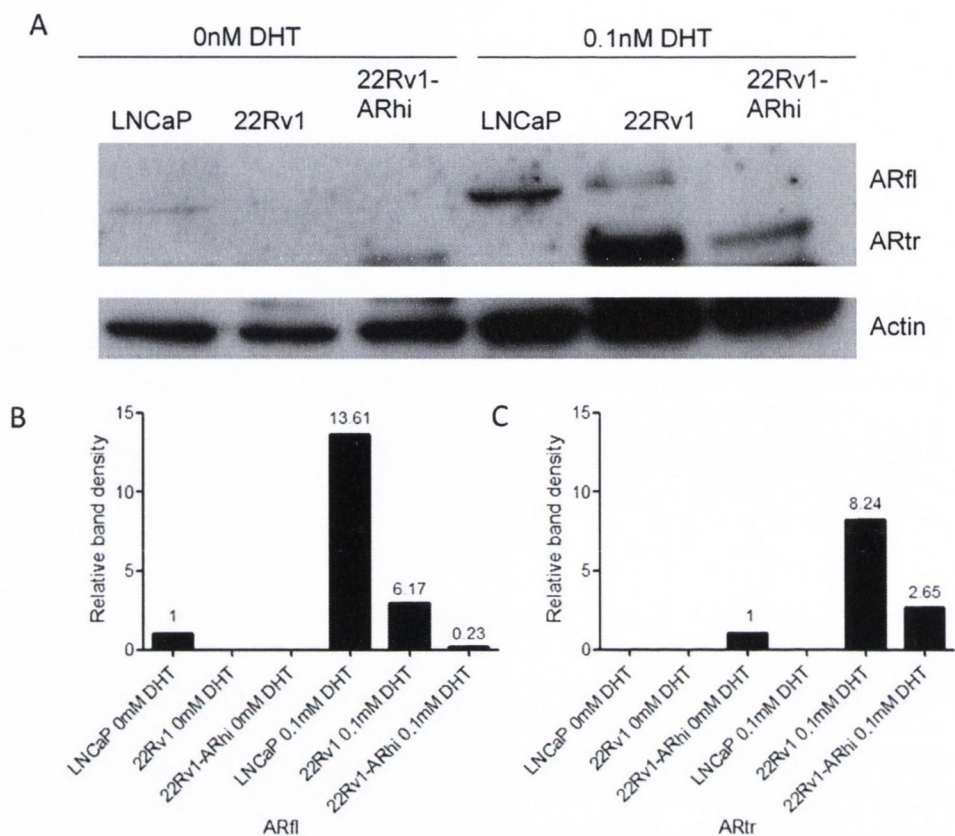


Figure 6.18: A) Nuclear protein expression levels of AR in LNCaP, 22Rv1 and 22Rv1-ARhi samples from untreated cells or cells treated with 0.1 nM DHT. Actin levels were used as a loading control. Representative blot, N=2. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

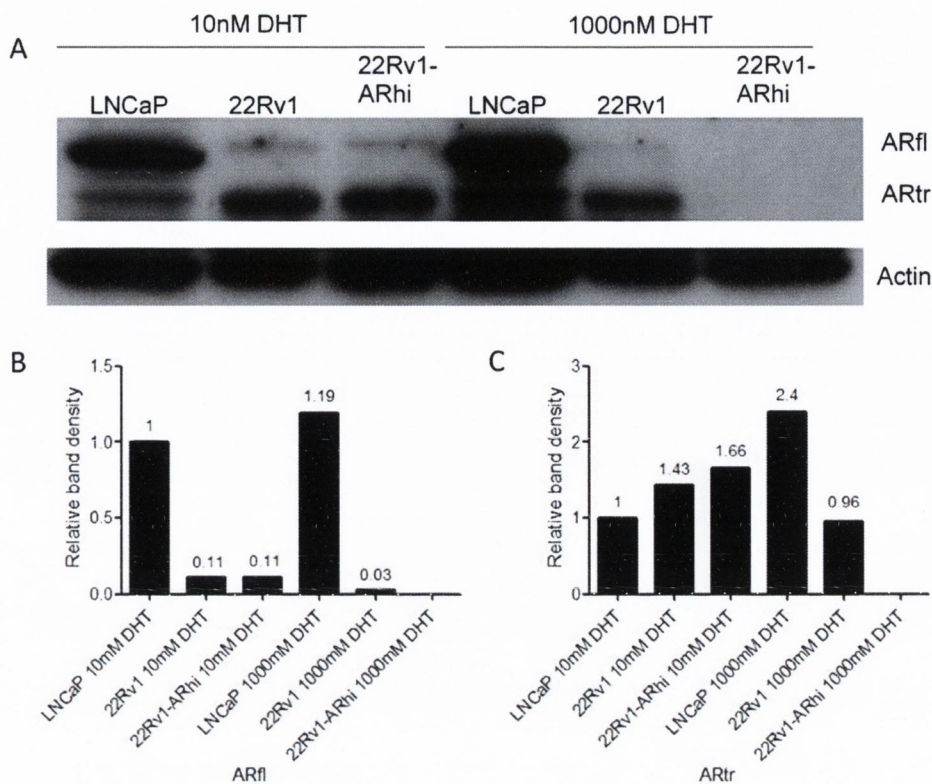


Figure 6.19: A) Nuclear protein expression levels of AR in LNCaP, 22Rv1 and 22Rv1-ARhi cell samples treated with 10 nM or 1000 nM DHT. Actin levels were used as a loading control. Representative blot, N=2. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

6.2.12 Calpain activation and inhibition

Although 22Rv1-ARhi cells are stably transfected with a vector that expresses full length wild type AR, both flAR and trAR overexpression was detected by Western blotting (section 6.2.3). One suggested mechanism for formation of trAR is post-translational cleavage of the protein by calpains. There is also evidence that the two isoforms of AR differ in some of the genes they regulate. Experiments were carried out using calpain activators and inhibitors to manipulate levels of trAR and assess if this had any impact on PSA expression levels.

22Rv1, 22Rv1-ARhi and LNCaP cells were treated with a calpain activator, ionomycin, and a calpain inhibitor, calpastatin, prior to protein extraction. Western blotting was carried out to determine if the levels of the truncated AR were altered

by activator/inhibitor treatment. Treatment with ionomycin had the desired effect, with an increase in the amount of the truncated AR in both aerobic (21% oxygen) and hypoxic (0.5%) cells in 22Rv1 (Figure 6.20) and 22Rv1-ARhi cell samples (Figure 6.21). Western blotting also demonstrated that treatment of the cells with the calpain inhibitor calpastatin resulted in a decrease in the expression levels of the truncated AR in 22Rv1 (Figure 6.22) and 22Rv1-ARhi (Figure 6.23), although this was only evident under hypoxic conditions.

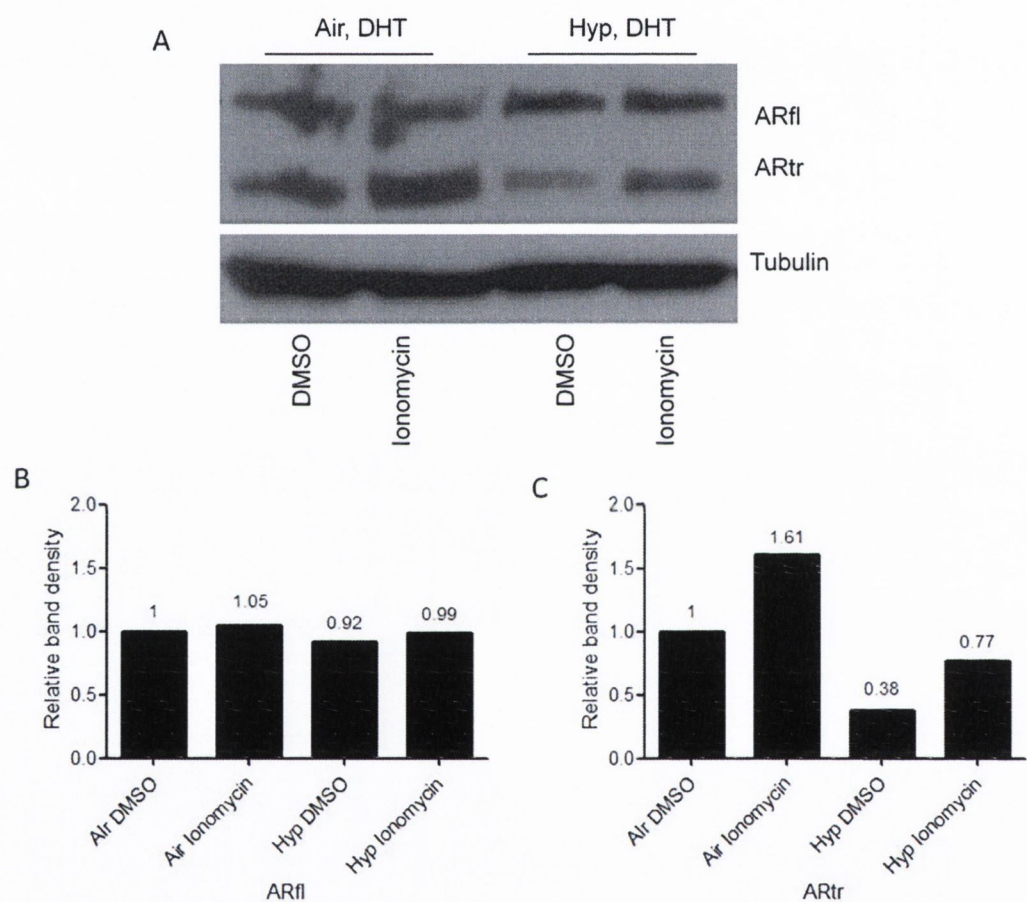


Figure 6.20: A) AR protein expression in 22Rv1 cells treated with the calpain activator ionomycin or DMSO as a control under aerobic and hypoxic conditions. Tubulin levels were used as a loading control. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

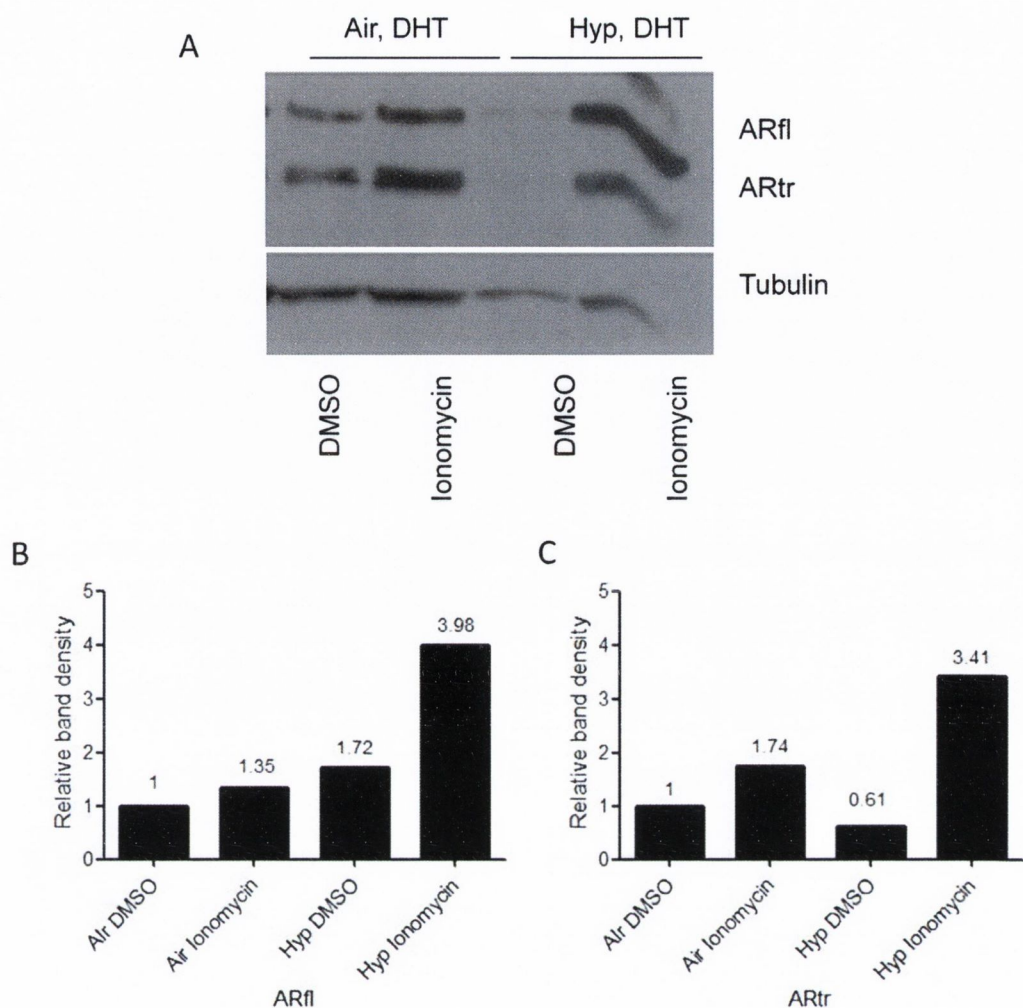


Figure 6.21: A) AR protein expression in 22Rv1-ARhi cells treated with the calpain activator ionomycin or DMSO as a control under aerobic and hypoxic conditions. Tubulin levels were used as a loading control. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

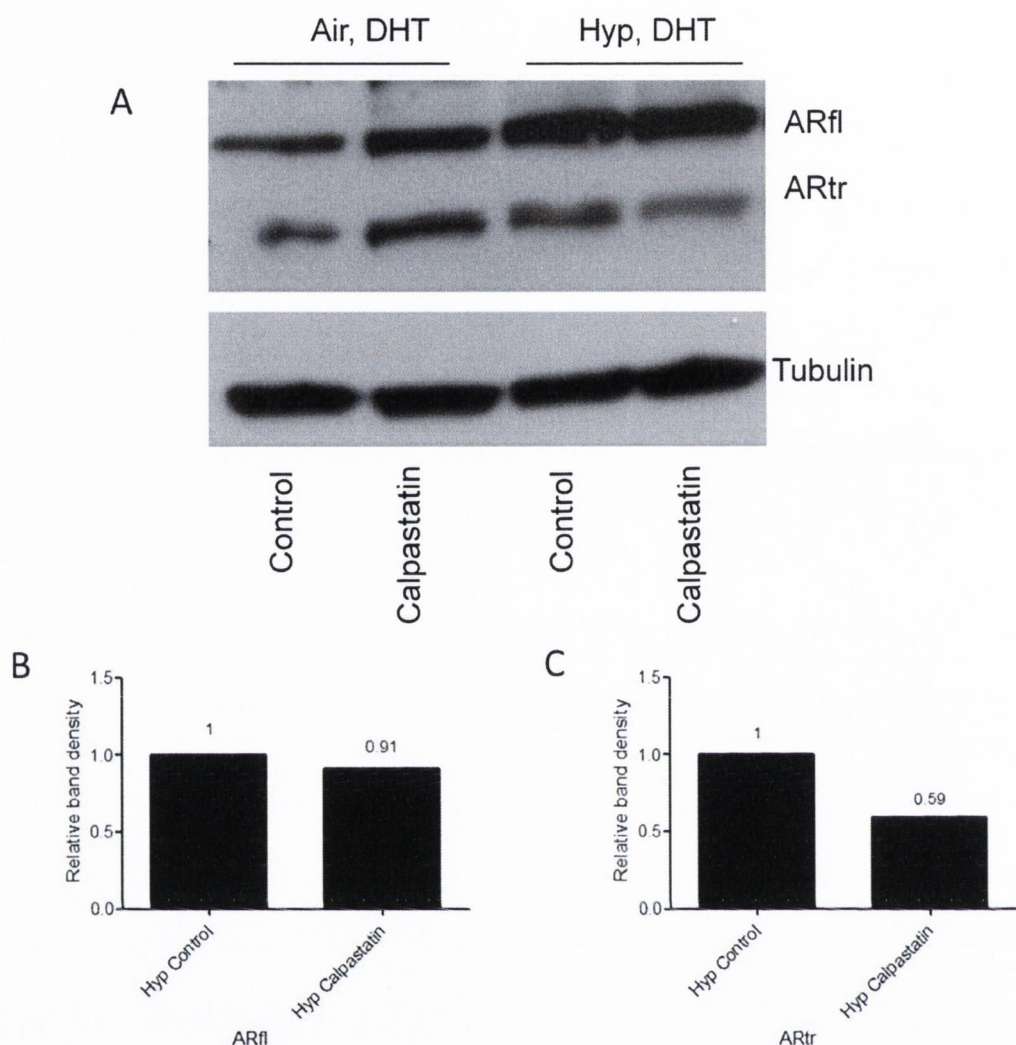


Figure 6.22: A) AR protein expression in 22Rv1 cells treated with the calpain inhibitor calpastatin or DMSO as a control under aerobic and hypoxic conditions. Tubulin levels were used as a loading control. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands, corrected against the density of the corresponding loading control band is displayed for ARfl (B) and ARtr (C).

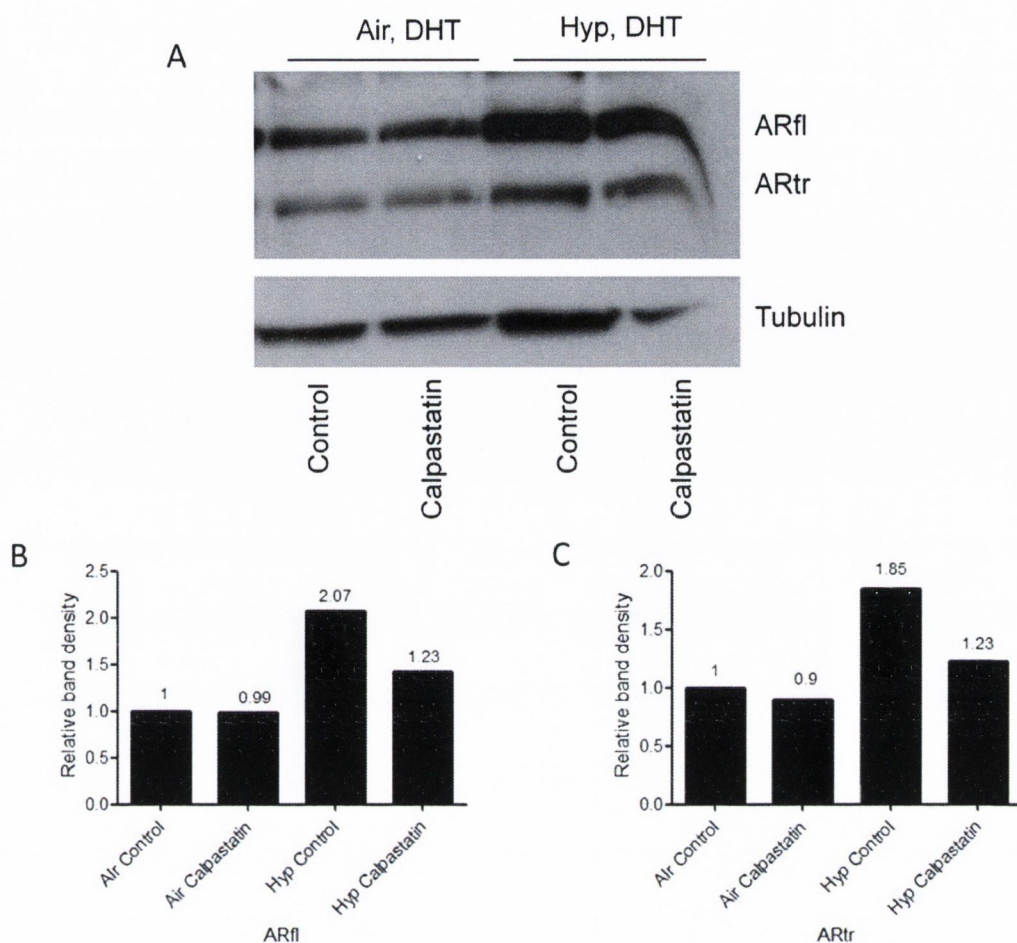


Figure 6.23: A) AR protein expression in 22Rv1-ARhi cells treated with the calpain inhibitor calpastatin or DMSO as a control under aerobic and hypoxic conditions. Tubulin levels were used as a loading control. ARfl, full length androgen receptor; ARtr, truncated androgen receptor. Densitometry analysis of the relative density of the protein bands for ARfl (B) and ARtr (C) (Due to the poor transfer of some of the loading control bands, this was not used to correct the results).

LNCaP, 22Rv1 and 22Rv1-ARhi were grown in androgen-depleted media for 72 hours prior to treatment with calpastatin (1 μ M) or ionomycin (10 μ M) or the equivalent control in DHT supplemented media under aerobic (21% oxygen) and hypoxic (0.5%) conditions. After a further 72 hours, media was collected for PSA EIA and an MTT assay was performed to normalise PSA levels to cell number. As was the case with previous experiments (Figure 6.6), there was a significantly lower level of PSA protein secreted by the 22Rv1-ARhi compared to 22Rv1 cells (Figure 6.24). Treatment of either cell line with calpastatin and ionomycin did not

significantly alter the levels of PSA compared to the equivalent control for each drug treatment. For 22Rv1 samples grown under hypoxic rather than aerobic conditions, PSA expression was significantly lower ($p < 0.001$) for calpastatin and its control and for the ionomycin control but not significantly different for ionomycin treated cells. For 22Rv1-ARhi only calpastatin treated cells were significantly different ($p < 0.001$) between aerobic and hypoxic samples. For LNCaP, there was no significant difference between calpastatin and ionomycin treated samples and their controls (Figure 6.25). PSA expression was higher under hypoxic conditions and this reached significance ($p < 0.05$) for calpastatin and ionomycin treated samples.

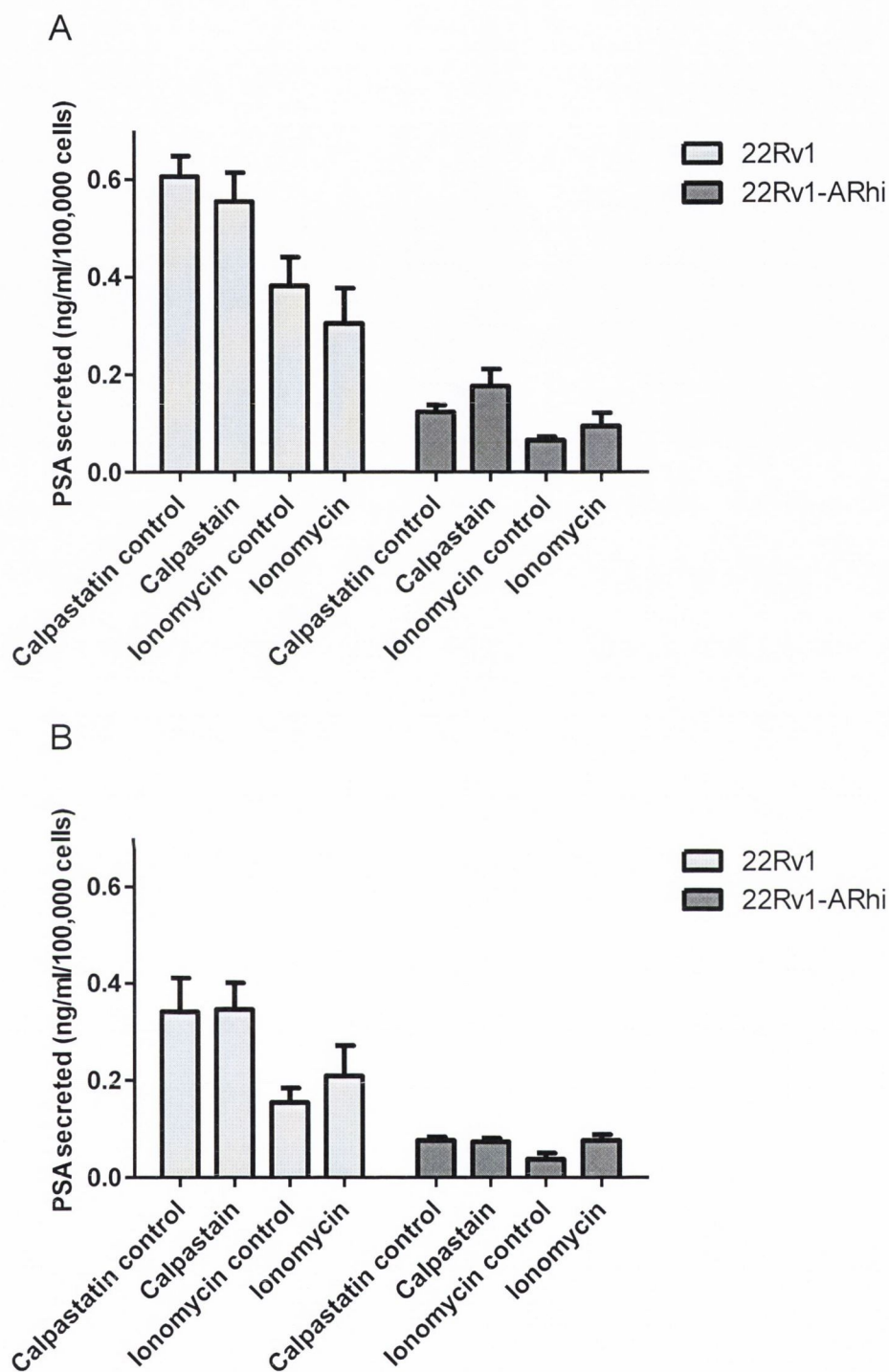


Figure 6.24: Secreted PSA levels following DHT (1000 nM) stimulation with calpastatin or ionomycin treatment in 22Rv1 and 22Rv1-ARhi under aerobic (A) and hypoxic conditions (B). Values represent the mean of three replicates $\pm$ standard error of the mean.

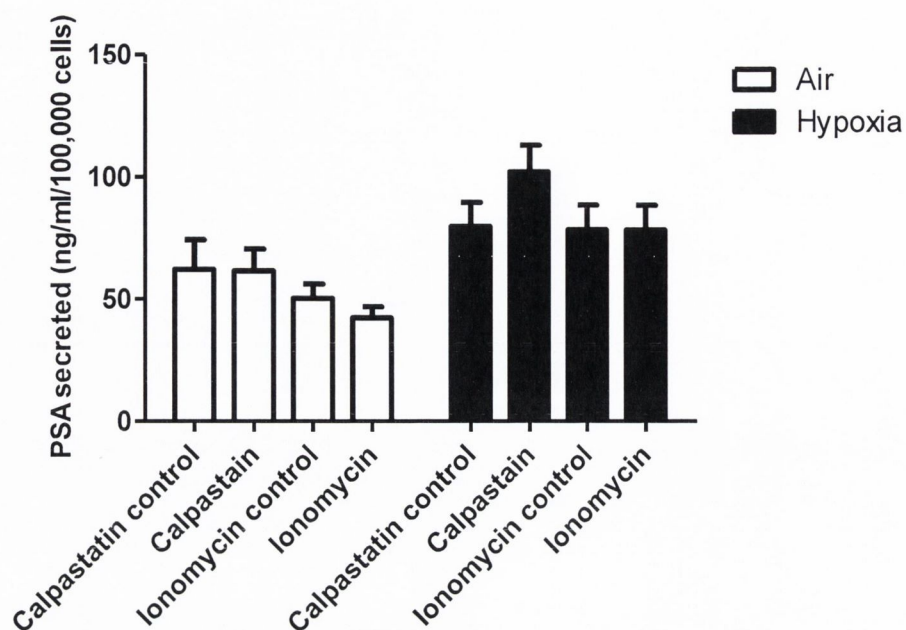


Figure 6.25: Secreted PSA levels following DHT (1000 nM) stimulation with calpastatin or ionomycin treatment in LNCaP under aerobic and hypoxic conditions. Values represent the mean of three replicates $\pm$ standard error of the mean.

6.3 Discussion

22Rv1 cells were stably transfected with pcDNA3.1-AR in an attempt to produce a primary prostate cancer cell line that would replicate the high levels of AR-responsive gene expression seen *in vivo*. After two rounds of cell cloning by serial dilution and growth of the transfected cells in Geneticin-containing media, the cells were tested to assess AR levels. Through Western blotting and qRT-PCR, it was confirmed that the stably-transfected cell line, designated 22Rv1-ARhi, had higher levels of AR expression than the parent cell line 22Rv1. However this did not translate into an increase in mRNA or protein levels of the AR-responsive gene PSA. In fact, expression levels of PSA were significantly lower in the transfected cell line, an unexpected result. It is unclear why this may be the case, although it is not unprecedented. Investigations by another research group of the VCaP prostate cell line compared the relative quantity of mRNA expression to LNCaP cells for nine AR-responsive genes.²³⁹ The basal level was markedly higher in VCaP for all the genes except PSA and S100P. The group suggested the gene *FKBP51* may be a better marker of androgen activity. Levels of FKBP51 mRNA were higher in VCaP than LNCaP with and without androgen treatment. Also, PSA expression showed little responsiveness to R1881, a synthetic androgen, in VCaP, but increased up to 6-fold in LNCaP. *In vivo*, in men treated with up to 9 months of androgen deprivation therapy, immunohistochemistry showed PSA protein expression was significantly decreased compared to tissue cores taken before treatment but it was still detectable and not eliminated by treatment.²⁴⁰ AR-regulated gene expression was examined; FKBP51 but not PSA expression was significantly downregulated in the treated tissue.

As an AR-responsive gene, FKBP51 mRNA levels were tested in 22Rv1-ARhi and compared to 22Rv1 for a range of DHT concentrations aerobic (21% oxygen) and hypoxic (0.5%) conditions (section 6.2.7). Although there was no significant difference in the expression of FKBP51 in 22Rv1-ARhi compared to 22Rv1, there was a trend towards increased expression for 22Rv1-ARhi with the lower concentrations of DHT (1 and 10 nM) as well as for 0 nM DHT under aerobic and hypoxic conditions. The much lower levels of expression observed for PSA in 22Rv1-ARhi were not replicated for FKBP51.

The levels of FKBP51 gene expression in 22Rv1 were induced much more strongly by DHT treatment than was earlier observed for PSA. Makkonen *et al.* obtained the same result in LNCaP and VCaP cells and demonstrated that it is because FKBP51 is controlled by AR at the level of transcription but PSA activation requires de novo protein synthesis.²⁴¹ In addition, the group identified 7 AREs within 4 major intronic sites downstream of the transcription start site of FKBP51. It would be interesting to test other known AR-responsive genes and compare their expression between 22Rv1-ARhi and the parent cell line. It is possible that the significant down-regulation in 22Rv1-ARhi may be specific to PSA.

There are mixed reports on whether androgen stimulation increases the proliferation rate of 22Rv1 cells. Attardi *et al.* showed treatment with DHT, testosterone, R1881 and other androgens over 72 hours resulted in a dose-dependent increase in cell number of 30-80%.²²⁵ Proliferation was inhibited by the anti-androgens OH flutamide or nilutamide. In contrast, Tepper *et al.* found no increase in proliferation rates when 22Rv1 were treated with 1 or 10 nM DHT compared to 0 nM and proliferation was, in fact, decreased by 32% with 100 nM DHT.³⁴

To determine if increased AR expression levels in 22Rv1 influenced the proliferation rate of the cells, they were seeded in androgen-depleted media with and without DHT for a period of 7 days before counting. The addition of 10 nM and 100 nM DHT increased cell number compared to those cells grown without DHT for both the 22Rv1 and 22Rv1-ARhi cells. This is in contrast to some previous reports, where DHT treatment did not affect cell number compared to cells grown in the absence of androgens. This increase only reached significance for 22Rv1-ARhi treated with 100 nM DHT ($p < 0.05$), perhaps indicating a more pronounced growth response to DHT in these cells. However, there was no significant difference between the cell numbers for the two cell lines at any of the DHT concentrations. A greater range of DHT concentrations were then used under both aerobic (21% oxygen) and hypoxic (0.5%) conditions using MTT assays to further test the growth rate of 22Rv1-ARhi compared to 22Rv1. One again there was not a significant difference between the proliferation rates of the two cell lines under any of the conditions tested.

Given the increase in cell proliferation of 22Rv1 and 22Rv1-ARhi after one week of DHT treatment, which reached significance for 22Rv1-ARhi with 100 nM DHT, it seems likely that stably transfected 22Rv1 with more pronounced AR overexpression would show increased growth rates in response to androgen treatment, compared to its parent cell line.

Despite the low levels of PSA expression in 22Rv1-ARhi, levels of CD expression in pPPE-CD transfected cells was of interest. As may have been expected, given the low levels of PSA promoter/enhancer activation (as indicated by PSA mRNA and protein levels), CD expression levels in these cells was lower than for 22Rv1. However, protein expression was still strong, and encouragingly, the patterns of upregulation seen in the parent cell line were also observed in 22Rv1-ARhi. Expression was higher for DHT treatment under hypoxic (0.5% oxygen) compared to aerobic (21%) conditions and radiation treatment with DHT resulted in increased CD levels compared to DHT alone. These results indicate that even when PSA activation levels are low, CD expression is robust and can be further upregulated by hypoxia and radiation.

In previous research, matched pairs of hormone-resistant and hormone-sensitive human prostate cancer xenografts were analysed using microarray and the only probe set found to be differentially regulated between all of the pairs was the one for AR cDNA.²⁴² AR upregulation in the hormone resistant xenografts was confirmed with Western blotting. Chen *et al.* then stably transfected LNCaP cells so that they expressed three times higher levels of AR (which was similar to the difference seen in the xenografts) and used clonogenic assays to demonstrate that higher AR expression allowed the cells to grow in 80% lower concentrations of R1881 compared to the parent cell line. Also, LNCaP-AR showed resistance to 10 μ M of the AR-antagonist bicalutamide. In fact, the antagonist activity of bicalutamide and other drugs cyproterone acetate and flutamide changed to weak agonist in the AR overexpressing cells.

Response to bicalutamide has previously been compared between LNCaP and VCaP to see if the increased AR protein expression in VCaP altered its effect on AR-activated gene expression.²⁴¹ In both cell lines bicalutamide was capable of inhibiting the androgen-induced expression of FKBP51, albeit slightly less

effectively in VCaP compared to LNCaP (80% vs. 90% inhibition). Although bicalutamide treatment in VCaP (but not LNCaP) resulted in AR binding to FKBP51 locus it did not induce binding of RNA polymerase II, so only resulted in minimal mRNA expression.

In a similar manner to the research by other groups described here, experiments set out in this chapter (section 6.2.10) tested if the higher levels of AR expression in 22Rv1-ARhi resulted in resistance to bicalutamide, using both MTT assays and clonogenic assays. However, the results of the MTT assays indicated that there was no decrease in the efficacy of bicalutamide in the 22Rv1-ARhi cell, despite the increased AR expression. This was confirmed with the results of the clonogenic assays. Despite an AR overexpression level similar to that obtained in LNCaP cells by Chen *et al.*, which resulted in bicalutamide becoming a weak agonist in that cell line ²⁴², there was no change in bicalutamide response in 22Rv1-ARhi compared to the parent cell line. It is possible that in 22Rv1, in which the AR upregulation of gene expression is weaker than in LNCaP, a more significant overexpression of AR would be required before an effect would be evident. Interestingly, the MTT assays indicated that bicalutamide treatment was less effective under conditions of 0.5% oxygen in all cell lines tested. It is possible that the increased AR activity observed in hypoxic cells ^{190, 211} is working against the AR antagonistic effects of bicalutamide.

22Rv1-ARhi cells were stably transfected with a plasmid containing the wild-type AR. Initial Western blotting experiments indicated that this resulted in an increased expression of the shorter isoform of AR, as well as the full-length protein. In fact, the ratio of trAR to flAR increased in 22Rv1-ARhi, with further dominance of the truncated protein. The unexpected finding that higher expression of AR in 22Rv1 does not translate to higher levels of PSA, an AR-activated gene, may reflect differential regulation of the gene by the two isoforms of the AR protein.

The full length and truncated ARs have distinct but overlapping target genes. Microarray analysis of 22Rv1 cells by Guo *et al.* identified 71 genes regulated by both AR isoforms, including *FKBP51*, but many well-known AR-regulated genes were not affected by AR3 knockdown (one of the main truncated isoforms), including PSA.²²⁷ However, the AR regulation of PSA is complicated as AR3

overexpression in LNCaP grown in androgen-depleted media resulted in a slight upregulation of PSA.²²⁷ AR3 can bind to the proximal but not the distal ARE in the PSA gene. Hu *et al.* found AR3 (in this case termed AR-V7) induced PSA reporter gene expression in AR3 transfected PC3 cells.²²⁸

The mechanisms that control the production of the different isoforms of AR, as well as the difference in their function have yet to be fully elucidated. Guo *et al.* suggested: “It is very likely that AR and AR3 (trAR) may function independently, cooperatively or competitively depending upon the context of promoters and cell types.”²²⁶ They demonstrated that knock-down of trAR in 22Rv1 cells did not affect the regulation of many established AR-controlled genes including *CLU*, *CLDN4* and *PSA*.

One mechanism thought to be involved in formation of trAR is post-translational cleavage of the AR protein by calpains. These calcium-dependent proteinases cleave in a specific manner based on sequence and protein conformation.²²⁹ Calpain activity is controlled by multiple processes including calcium levels, phosphorylation and levels of the inhibitor calpastatin. Research by other members of the Prostate Molecular Oncology Research Group (unpublished) has shown an increase in calpain 2 levels in 22Rv1 cells grown in hypoxic conditions (0.5% oxygen) compared to aerobic cells (21%). In this chapter, a known calpain activator (ionomycin) and inhibitor (calpastatin) were used in an attempt to alter the ratio of trAR to flAR and subsequently examine if this influenced PSA expression.

Western blotting experiments demonstrated that trAR protein levels were increased in both 22Rv1 and 22Rv1-ARhi cells treated with ionomycin under aerobic (21% oxygen) and hypoxic (0.5%) conditions. A decrease in trAR levels was detected with calpastatin treatment in both cell lines, although this was evident only for the hypoxic samples. These changes in trAR levels did not translate into changes in PSA protein levels however. Although differences in trAR expression were observed with Western blotting, concurrent changes in the level of flAR were not obvious and may have been necessary to affect PSA secretion levels. It may be informative to test expression levels of other AR-responsive genes in these cells lines in response to calpain inhibition and activation. *FKBP51* was one of the genes found to be responsive to both flAR and trAR by Guo *et al.*²²⁷ An overlapping but

distinct set of genes regulated by the two isoforms of AR may explain why FKBP51 levels were maintained while PSA levels were significantly lower in 22Rv1-ARhi, in which trAR appears to dominate more than in the parent cell line.

The study of the mechanisms that result in the formation of different isoforms of AR is clinically relevant. In BPH tissue only small amounts of trAR are detected and mainly found in the basal and stromal cells. In PIN lesions and malignant tissue, the levels increase in the luminal cells, suggesting trAR may play a role in development and progression of the tumour.^{226, 227, 229} A role for calpains in cancer progression is supported by the detection of increased levels in prostate tumours as well as other tumour types, with highest levels in metastatic tissue.^{229, 243} The research in this chapter, demonstrating the increase/decrease in trAR with the treatment of 22Rv1 cells with a calpain activator/inhibitor, supports previous research demonstrating AR is a calpain substrate.²²⁹ Additional study of 22Rv1-ARhi may help further the understanding of the formation and function of these truncated AR isoforms.

To gain insight into AR action in 22Rv1 and 22Rv1-ARhi, nuclear protein was extracted to compare AR levels between the two cell lines using Western blotting. Protein was also extracted from LNCaP cells to use as a control. When the cells were grown in the absence of DHT in androgen depleted media, trAR but not flAR was detected for 22Rv1-ARhi. trAR lacks a ligand binding domain and may be able to enter the nucleus in the absence of androgen. It is possible that nuclear trAR levels for 22Rv1 were not high enough to be detected. Samples were treated with increasing concentrations of DHT. For 0.1 nM DHT, nuclear levels of both full-length and truncated AR were, in fact, higher for 22Rv1 than 22Rv1-ARhi. The levels for the two cell lines were similar at 10 nM DHT but at 1000 nM the amount of AR in the nucleus seemed to decrease dramatically for 22Rv1-ARhi. The amount of flAR was highest for LNCaP cells at all DHT concentrations, as would be expected. The low levels of nuclear AR for 22Rv1-ARhi cell samples compared to 22Rv1 may go some way to explain why the levels of PSA expression are so much lower in the stably transfected cell line, despite the increase in overall AR protein levels.

As mentioned, the level of trAR is increased in malignant compared to BPH tissue. However, while the tumour is hormone-naïve, the majority of the protein remains in the cytoplasm but over 40% of CRPC tumours will have trAR in the nucleus.²²⁷ Guo *et al.* suggest that nuclear localisation of trAR may still be tightly regulated, despite the lack of a LBD, although the mechanisms controlling it have yet to be elucidated.²²⁶ One suggestion is phosphorylation by Src tyrosine kinase, which would still be possible in trAR as the phosphorylation sites are located in the NTD.^{226, 244} Src inhibitors have been shown to stop transport of trAR into the nucleus in CWR-R1 cells.²²⁶

There is still much to learn about the origin and activity of the truncated AR but it is a worthwhile endeavour due to its clinical relevance and likely role in the progression to androgen independence. Depending on the environment, it is possible that trAR functions independently or in combination/competition with flAR.²²⁶ It appears that the 22Rv1-ARhi cell line may be useful for studying this form of the AR protein.

This research involved the successful creation of an AR overexpressing 22Rv1 cell line. However, subsequent characterisation of the cell line and the finding that PSA expression levels were in fact significantly lower than in the parent cell line, highlights the complex nature of AR gene regulation. The cell line was still useful for analysis of pPPE-CD activity, demonstrating its utility in a low PSA activation levels. Experiments were also carried out in an attempt to understand the mechanisms resulting in decreased PSA expression. 22Rv1-ARhi shows a higher level of trAR expression than might have been expected, pointing to the complicated nature of the pathways which ultimately determine AR protein size. Much remains to be learned about the field and further study involving 22Rv1-ARhi may be useful in the quest to further understand AR gene regulation and its role in development and progression of prostate cancer.

Chapter 7

Development of a spheroid model
of prostate cancer and its use to
evaluate the activity of pPPE-CD

Chapter 7 Development of a spheroid model of prostate cancer and its use to evaluate the activity of pPPE-CD

7.1 Introduction

Tumour cell culture typically involves the growth of cells as a monolayer on the surface of a flask. Analysis of these cells increases our understanding of tumour biology and can be used to test the potential of various treatment strategies. However, it is now established that although informative, this artificial environment is not an ideal representation of an *in vivo* tumour.²⁴⁵⁻²⁴⁷ The growth of tumour cell lines in this manner results in the loss of some characteristics and the exaggeration of others. For example, cell-cell communication, polarization and differentiation are reduced while proliferation and inflammatory responses are enhanced.²⁴⁸ Crucially, this could have a significant impact on the drugs and treatments that are effective in two-dimensional (2D) cell culture; those that target proliferation and mitosis will be effective but not those that affect cell-cell interaction or maturation, for example. Their efficacy *in vitro* may not reflect how these drugs will behave *in vivo*.

For many years it has been recognised that a useful alternative to the typical culture of cancer cells as an adherent monolayer is their growth in a three-dimensional (3D) cell system as multicellular tumour spheroids, which are spheres of densely packed cells.²⁴⁹ This system has advantages over 2D cell culture and has potential to bridge the gap between *in vitro* cell growth and animal models. Any improvement in the artificial representation of tumour behaviour *in vitro* would be advantageous and would improve the ability to assess the potential of a new drug or treatment strategy early in its development. The capacity to rule out potential drugs using cell culture reduces costly, time-consuming research using *in vivo* tumour models. It would also be advantageous to drug screening experiments if the 3D cell culture technique was suitable for high throughput techniques and compatible with automated liquid handling systems.²⁴⁷

Various groups have compared the gene expression profiles of spheroids and the equivalent cells grown as a monolayer culture in different tumour types, for example, in ovarian²⁵⁰, colon²⁵¹ and hepatocellular carcinoma cell lines.²⁵² There

are differences between cells grown in 2D and 3D culture in genes that regulate proliferation, differentiation and treatment survival and resistance. Importantly, gene expression in the spheroid models has been demonstrated to be closer than monolayer cell culture to that observed *in vivo*. In the case of prostate cancer, Harma *et al.* grew a range of cell lines in Matrigel and found that approximately 3400 mRNAs were differentially regulated in spheroids compared to monolayer cultures.²⁴⁸ Genes involved in mRNA processing, mitochondrial and ribosomal activities and metabolic processes were downregulated reflecting the reduction in growth and proliferation in spheroids.

There are a variety of different techniques available for spheroid formation. For large scale production of numerous spheroids, a spinner flask can be used to prevent adherence to the flask surface and maintain an even distribution of nutrients. Similar approaches involves roller flasks or gyratory shakers.²⁴⁶ A disadvantage of this type of rotating cell culture is the use of large amounts of media which would necessitate high levels of the drug of interest to achieve the desired concentration. In the liquid overlay method, growth of spheroids on a smaller scale can be achieved by coating the bottom of a plate with agar before seeding the cells. This method has been shown to result in spheroids of very consistent size but a disadvantage is the labour burden associated with individually coating each well before use.²⁴⁶ The hanging-drop method prevents cell-plate interaction by pipetting a drop of cell suspension on to a plate surface which is then turned upside down. This is only suitable for short-term culture of spheroids although Tung *et al.* recently developed a 384-well culture plate for hanging-drop spheroid formation which allows for the use of liquid-handling machines and long-term growth of spheroids.²⁴⁷

At a sufficient diameter (200 – 500 μm) spheroids are large enough to reduce the diffusion of glucose and oxygen resulting in a gradient in concentration from the edge to the centre.^{245, 249} Morphological characteristics of spheroids typically involve a rim of healthy, proliferating cells (modelling cells in a tumour which have access to a blood supply) surrounding a core of quiescent cells which will become necrotic over time.²⁴⁶ Spheroids can act as an *in vitro* model of micrometastases or regions of solid tumour lacking adequate vascularisation.²⁵³

The efficacy of various drugs has been compared between cancer cell lines grown as adherent cells and as spheroids. For example, Mellor *et al.* examined a range of chemotherapeutic drugs in a study of drug resistance in a colon adenocarcinoma cell line.²⁵⁴ Growth of these cells as spheroids reduced proliferation and increased levels of the quiescence marker p27(kip1). They found that in comparison to the 2D model, in 3D, cell death after doxorubicin or cisplatin treatment was reduced and was lost for 5-FU and vinblastine. Similar results were observed by Friedrich *et al.* testing 5-FU, Irinotecan, and C-1311 using the acid phosphatase assay in HT29 and HCT-116 cells.²⁵⁵ Variation in the efficacy of 5-FU was observed between 2D and 3D cultures of A431.H9 cells.²⁴⁷ At a concentration of 10 μ M, viability was reduced by 95% in monolayers but only by 25% in spheroids. Tung *et al.* suggest that because 5-FU targets proliferating cells, it will cause less damage to the slower growing cells within spheroids and may also have difficulty penetrating the spheroid. Spheroids have also been used to test combination treatments involving radiation and drugs and/or radiosensitizers, which is of interest for this research. For example, in HepG2 spheroids (a hepatocellular carcinoma cell line), supra-additive effects on spheroid outgrowth and toxicity were measured when 10 Gy radiation was combined with cisplatin, gemcitabin or 5-FU.²⁵⁶

7.1.1 Aims and objectives

This research involves the use of a novel technique for 22Rv1 spheroid formation. It is a result of collaboration with Dr. Anthony Davies, Head of Irish National Centre for High Content Screening and Analysis in Trinity College Dublin and Scientific Director of Biocroí Ltd, where an advanced suspension media, Happy Cell (HC) has been developed. It was hypothesised that this would allow for the generation of spheroids and this chapter discusses the optimization of this technique and subsequent spheroid characterization. This model may reflect better the range of environmental conditions observed within an *in vivo* tumour and affords the opportunity to explore the effects of gene therapy in an *in vitro* system closer to the *in vivo* comparison.

Aim: Optimise formation of 22Rv1 spheroids using HC system.

Aim: Confirm formation of spheroids and characterise morphology.

Aim: Use the spheroids as a model to assess pPPE-CD activity under conditions investigated in 2D culture in chapters 4 and 5.

7.2 Results

7.2.1 Optimisation of spheroid formation

Following optimisation of cell number, initial experiments led to the formation of spheroids in both 96- and 24-well plates. However, a percentage of the 22Rv1 cells did not remain in suspension and stuck to the plate surface (Figure 7.1). To try to remedy this, a layering technique was attempted during cell seeding. 100 μ l of HC media was placed in the wells of a 96-well plate, followed by another 50 μ l of cell containing HC media, which was gently layered on top. As a control, in another set of wells, the second lot of media was mixed with the first. Concentrations of 4×10^4 – 8×10^4 cells/well were found to be optimal. This layering technique greatly reduced the number of adherent cells and therefore resulted in increased spheroid number (Figure 7.2). However, it did not eliminate the problem. Subsequently, spheroids were grown in ‘Hydrocell’ low cell binding plates (Nunc) and this overcame the problem of concurrent spheroid and adherent cell growth (Figure 7.3).

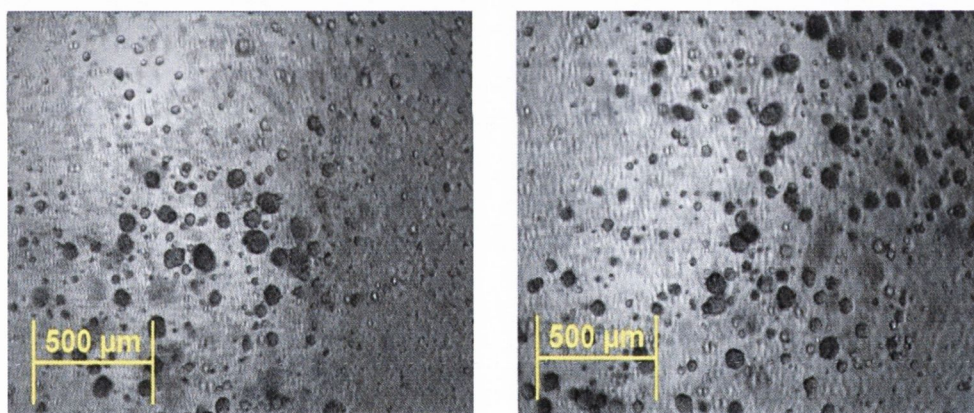


Figure 7.1: 22Rv1 cells seeded in HC media demonstrating the combination of spheroid formation and adherence of cells. Bright field images taken using an IN cell Analyzer 1000 at $4 \times$ magnification.

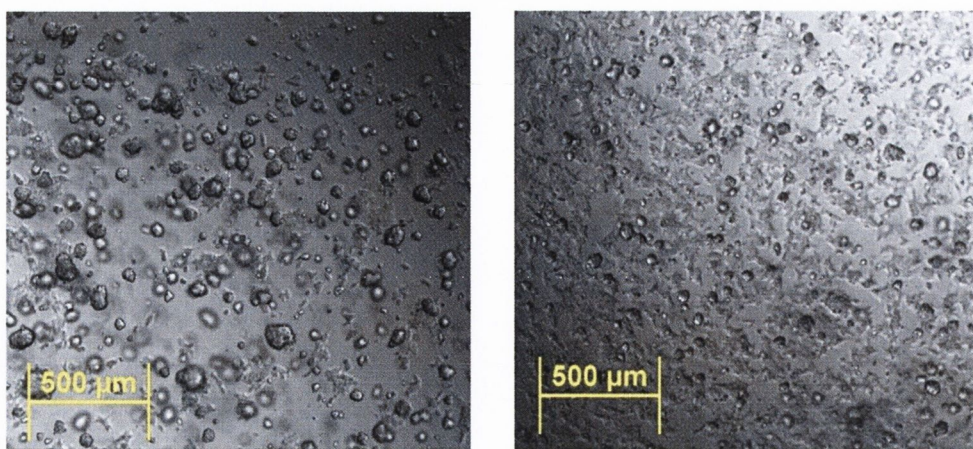


Figure 7.2: Left: 22Rv1 cells seeded in HC media using a ‘layering’ technique to reduce cell adherence. Right: a well in which the cells were mixed upon addition to HC media. Bright field images taken using an IN cell Analyzer 1000 at 4 × magnification.

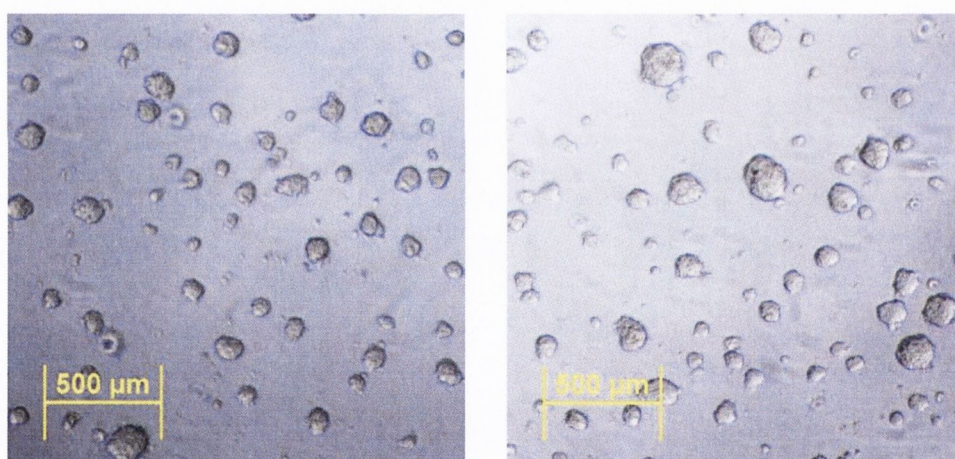


Figure 7.3: 22Rv1 cells seeded in HC media in ‘Hydrocell’ 96-well plates. Bright field images taken using an Olympus CKX41 fluorescent microscope at 4 × magnification.

7.2.2 *Spheroids vs. aggregates*

One distinguishing feature of spheroids as opposed to cell ‘aggregates’ (loose bundles of cells) is their robustness.²⁴⁵ 22Rv1 cells were transfected with a GFP-expressing vector before being seeded in a 24-well plate in HC media. 3 days later, a p1000 pipette was used to transfer 100 μl of spheroid containing media to the

wells of a fresh 96-well plate which was then imaged with the IN Cell Analyzer (Figure 7.4). This demonstrated that the spheroids can be manipulated and remain intact when transferred from well-to-well.

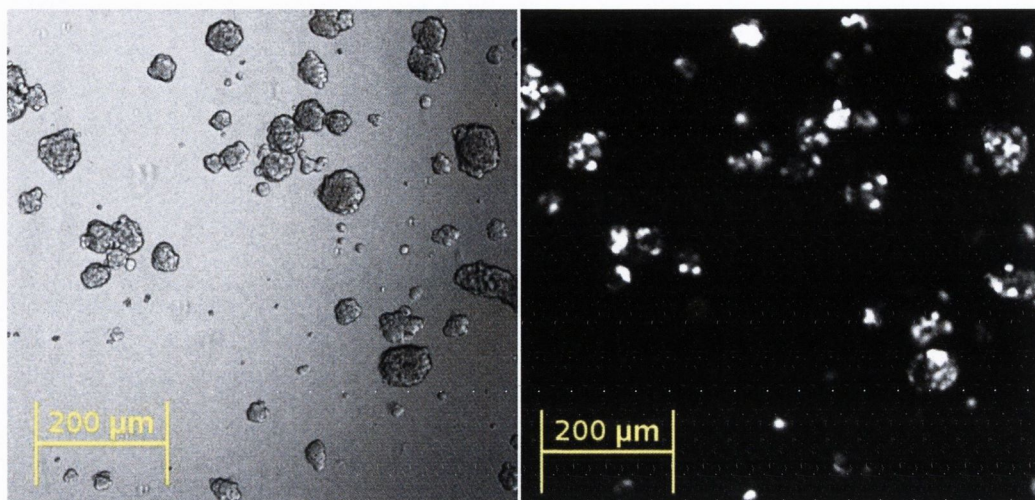


Figure 7.4: GFP-transfected 22Rv1 spheroids transferred from a 24-well to 96-well plate before imaging using high-content screening an IN cell Analyzer 1000 at 10 × magnification on the bright field setting (left) and fluorescent setting (right)

7.2.3 *Morphological assessment of spheroids*

Following optimisation, and successful spheroid formation, confocal microscopy was used to examine the spheroids using the fluorescent dyes Hoechst and PI. Hoechst, a nuclear stain, showed intact nuclei and confirmed that the cells were healthy and not clumps of dead cells (Figure 7.5). PI is a membrane impermeant dye that binds to DNA and can be used to detect dead cells. PI staining was mainly restricted to the spheroid core (Figure 7.6).

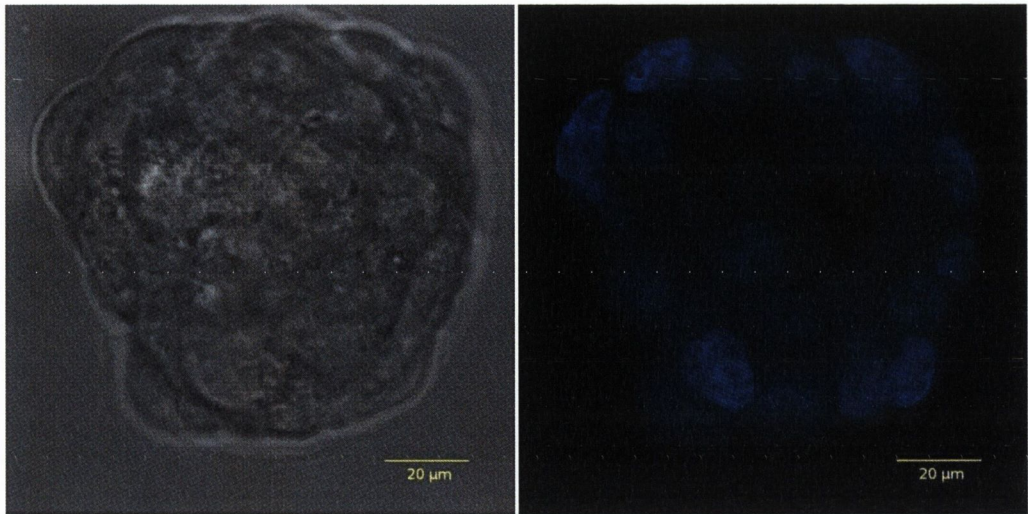


Figure 7.5: A representative spheroid stained with Hoechst and imaged at 63 × magnification using confocal microscopy on the bright field setting (left) and fluorescent setting (right).

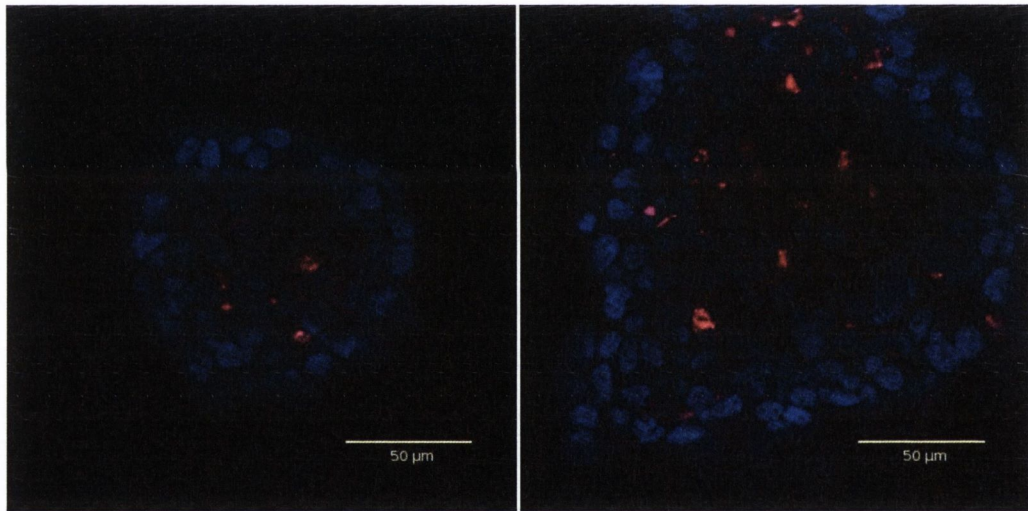


Figure 7.6: Representative spheroids stained with Hoechst and PI at 40 × magnification using fluorescent confocal microscopy.

7.2.4 Mechanism of spheroid formation

To try to elucidate whether spheroid formation is a result of cell division or aggregation, the 22Rv1 cells were seeded in HC media in 96-well plates, imaged immediately (Day 0) and then tracked over a number of days using the Innovatis Cellavista imaging machine (Figure 7.7). From these results it appears that initially, between time of seeding and 24 hours later (Day 1), the cells aggregate to form

clumps. However the resulting spheroids increase in size between day 2 and 5, suggesting there is subsequent cell division within spheroids.

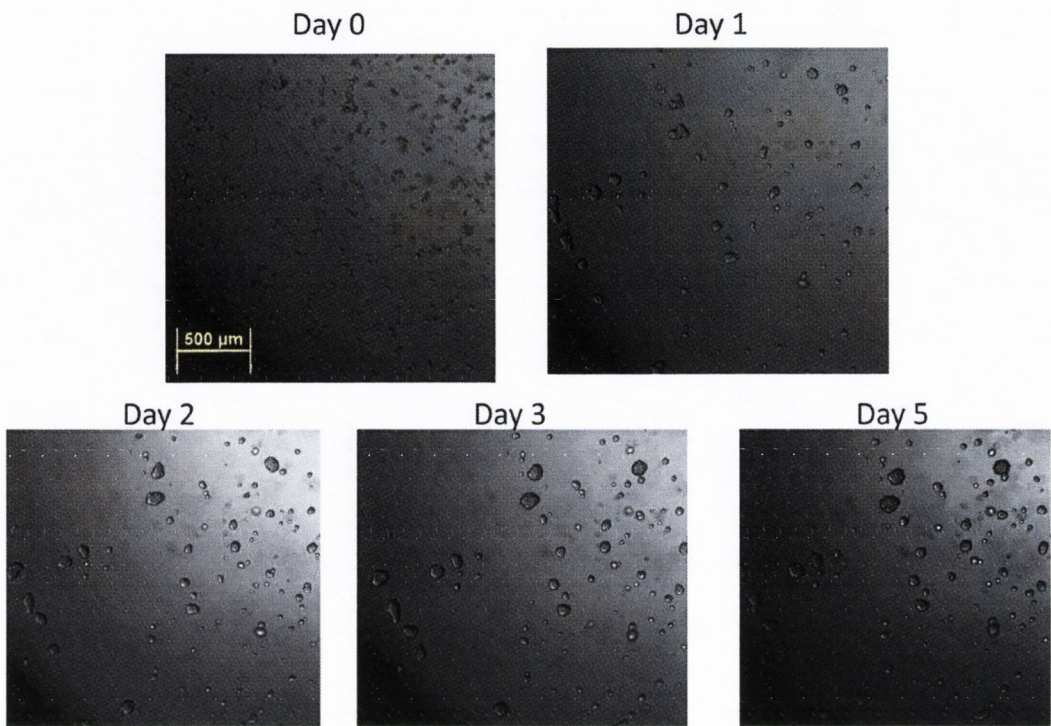


Figure 7.7: 22Rv1 cells seeded in HC media in a 96-well plate and imaged at 4 × magnification over 5 days using the bright field setting on the Cellavista Analyzer.

7.2.5 Potential for use in high-throughput technology

An experiment was carried out using the Echo Liquid Handling System to examine the potential for the use of HC media with automatic liquid handling robots. An equal number of Hoechst-stained cells in HC media or normal DMEM media was placed in the source well of the Echo and seeded in a 384-well plate at different time points before cell counting using high-content screening . This was carried out to compare the consistency in cell numbers over time, which can change due to sinking of the cells in the source well. For HC media there was a steady high level of cell seeding at 1, 5 and 10 minutes with a slight drop off at 20 minutes (Figure 7.8). The levels of cell seeded for DMEM media was much lower and decreased with successive time points so that there were very few cells in the wells seeded after 20 minutes. These results suggest HC media could help avoid the problem of cells sinking in the original cell suspension, which can affect cell seeding density

over time when automated liquid handling systems are used.

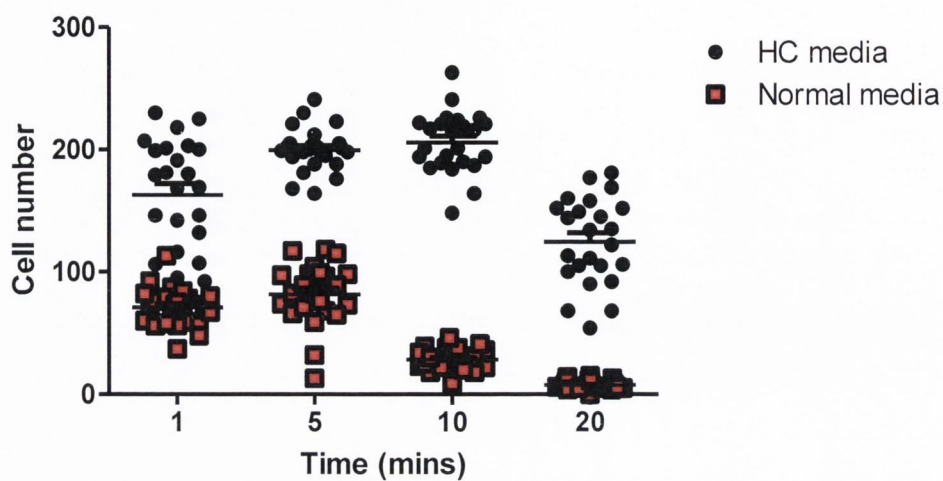


Figure 7.8: Cell number per well after automatic liquid dispensing at different time points using a cell suspension of HC media and normal DMEM media.

The possibility of high-throughput testing using the spheroid model was further explored. ‘Nanowells’ were used and the cells were dispensed in 200 nl of HC media using the Deerac Fluidics Equator Eight-Tip Pipetting System (Figure 7.9). The technique was optimised for cell number as well as various other parameters. Nanowells show the potential of this technique to be used in high-throughput setting and demonstrate spheroid formation using cells dispensed by liquid handling robots.

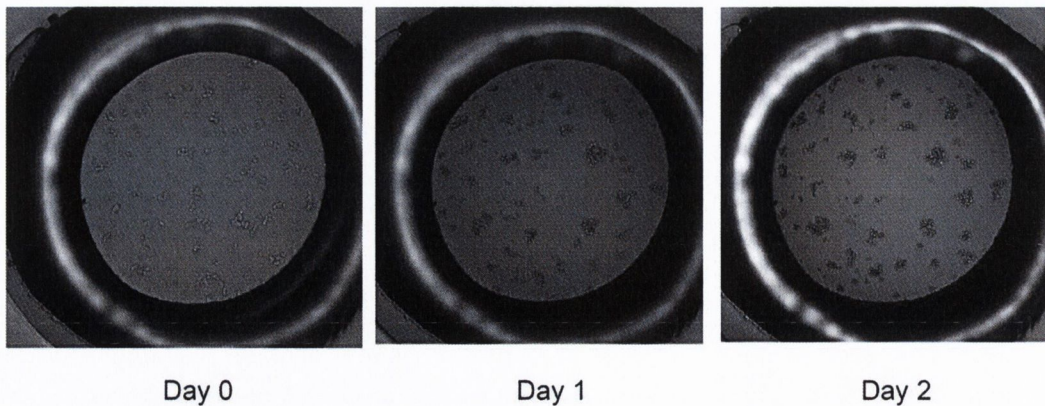


Figure 7.9: 22Rv1 cells in a well of a nanowell slide, imaged over the course of 3 days using the bright field setting on a Cellavista Analyzer at 4 × magnification.

7.2.6 *PSA EIA*

A PSA EIA was used to compare the levels of PSA secretion from the adherent 22Rv1 cells and those grown as spheroids. A third group that was tested was 22Rv1 cells that were allowed to adhere to the plate before the normal DMEM media was replaced with HC media. This was to control for any possible influence the HC media itself may have on PSA secretion. The results of the PSA EIA showed that the level of PSA secreted from the spheroids is significantly higher than for the adherent cells (Figure 7.10). In the absence of DHT there are detectable amounts of PSA in the media which are significantly higher for the spheroids ($p < 0.05$). When the cells are stimulated with 1000 nM DHT, the levels of PSA increase for all cell types but, again, are significantly higher for the 22Rv1 spheroids ($p < 0.05$).

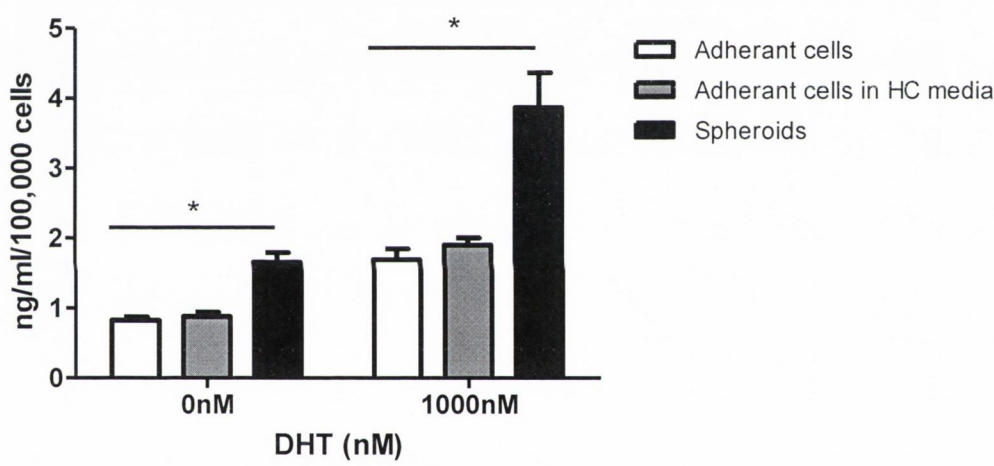


Figure 7.10: PSA protein levels following DHT stimulation in 22Rv1 adherent cells and spheroids. Values represent the mean of three replicates $\pm$ standard error of the mean. *, $p < 0.05$.

7.2.7 *GFP transfection*

Before pPPE-CD transfection of 22Rv1 and the growth of these cells as spheroids, a GFP-expressing vector was used to test whether the transfected cells would form spheroids and how long the expression would last. 22Rv1 cells were transfected with the vector and the next day were seeded in a 96-well plate (normal or Hydrocell). The cells were tracked over the course of a week. The transfection rate was approximately 50%. Cells grown in HC media formed spheroids and the GFP expression remained strong throughout the course of the experiment (Figure 7.11).

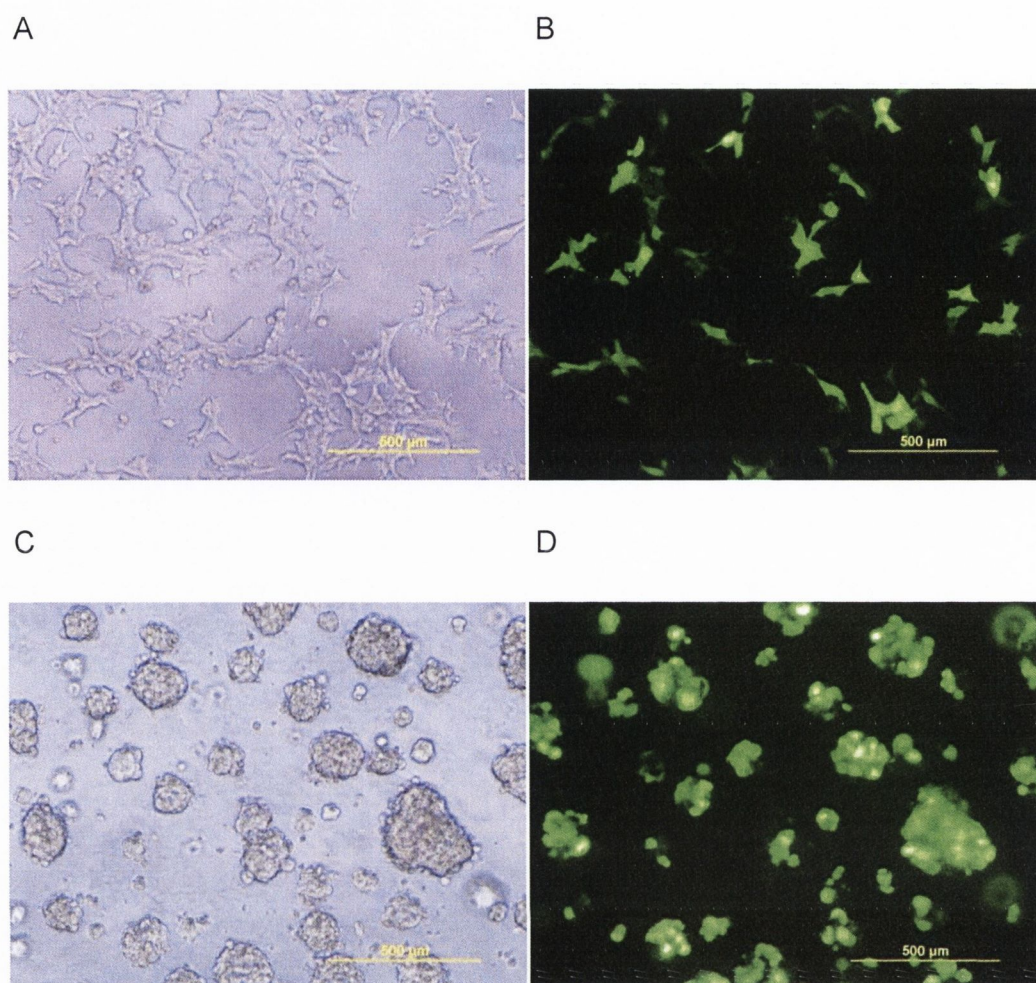


Figure 7.11: GFP-transfected 22Rv1 cells grown as adherent cells (A & B) and spheroids (C & D) 5 days after seeding. Images were taken with an Olympus CKX41 fluorescent microscope at 20× magnification using brightfield (A & C) or fluorescent (B & D) microscopy. Scale bar represents 500 μm.

7.2.8 Cell viability following pPPE-CD/5-FC treatment

Following characterisation, spheroids were used as a model to further test the activity of pPPE-CD. 22Rv1 cells were transfected with pPPE-CD or the constitutively active vector, pCMV-CD, or mock transfected as a negative control. The following day the cells were reseeded for growth as spheroids. Cells were also grown as an adherent monolayer and treated in the same manner as the spheroids throughout the experiment to compare the two models. The cells were subsequently treated with DHT and/or 2 Gy radiation under aerobic (21% oxygen) or hypoxic (0.5%) conditions and three days later with 5-FC. The cells were stained with

Hoechst and high-content screening was used to assess the efficacy of pPPE-CD/5-FC treatment.

Adherent 22Rv1 cells

For the adherent 22Rv1 cells, the high-content images were analysed to measure cell number, which were compared between those treated with 5-FC and those without for the different transfectants under the various conditions. The results were normalised against the untransfected cells not treated with 5-FC under each of the growth conditions. For those cells grown in air with and without DHT, there was no significant difference for untransfected cells or those transfected with pPPE-CD or pCMV-CD (Figure 7.12). However, when radiation was combined with DHT stimulation under aerobic conditions, the decrease in cell number for the pPPE-CD transfected cells treated with 5-FC, compared to untreated was significant ($p < 0.01$, Figure 7.13). This significant change in cell number in response to 5-FC treatment was not observed for the untransfected cells. Radiation treatment alone, without DHT, was not sufficient to achieve significant cell kill in pPPE-CD-22Rv1 (Figure 7.13).

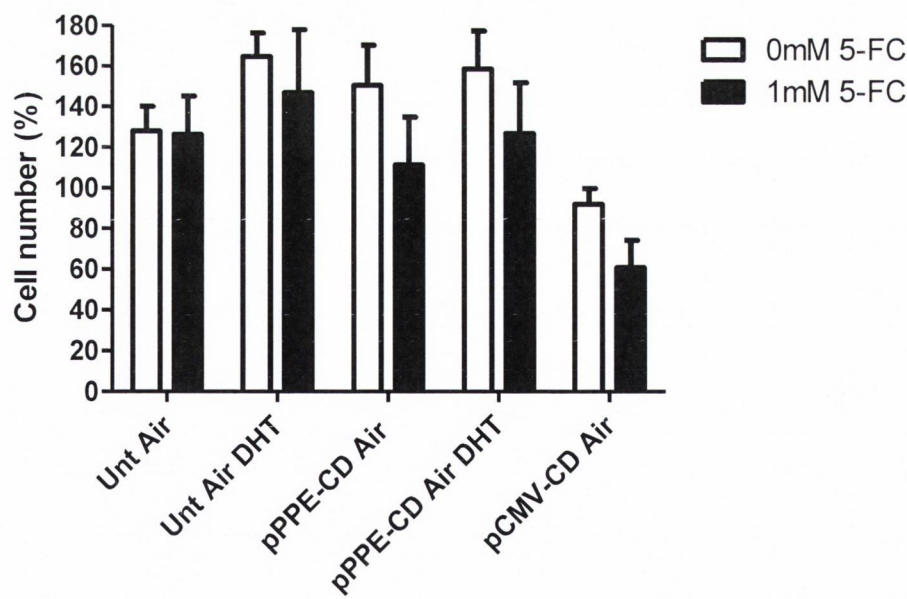


Figure 7.12: 22Rv1 cell count after treatment in air with and without DHT (1000 nM). Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected.

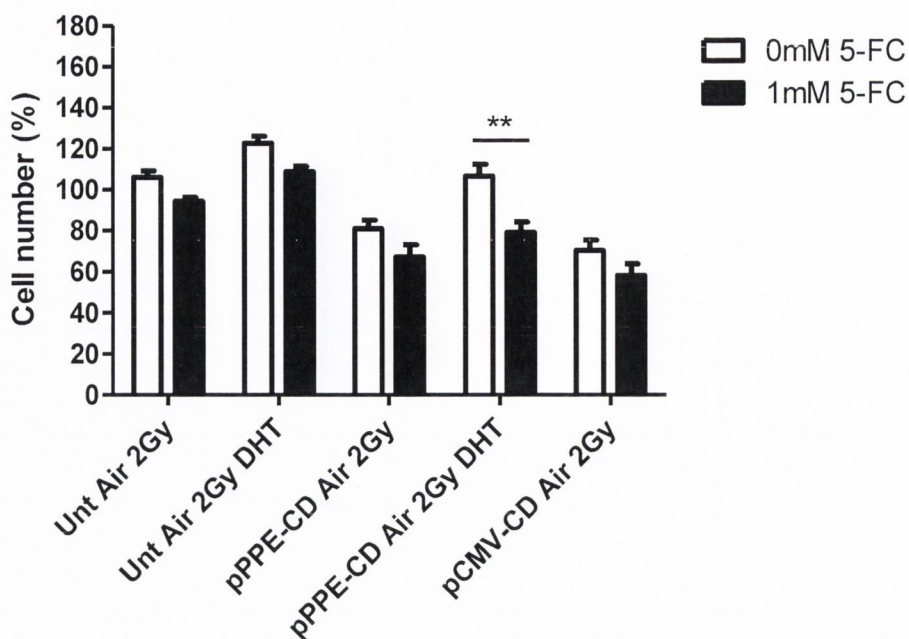


Figure 7.13: 22Rv1 cell count after treatment in air with radiation and with and without DHT (1000 nM). Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected; **, $p < 0.01$.

The results for cell number were compared between those pPPE-CD transfected cells, treated with and without radiation (using one way ANOVA with Bonferroni multiple comparison testing) to assess if there was increased efficacy of the pPPE-CD/5-FC system when it is combined with radiation. It was shown that both 1 mM 5-FC and radiation treatment decrease cell survival (with and without DHT) but the decrease is even greater when the two treatments are combined (Figure 7.14).

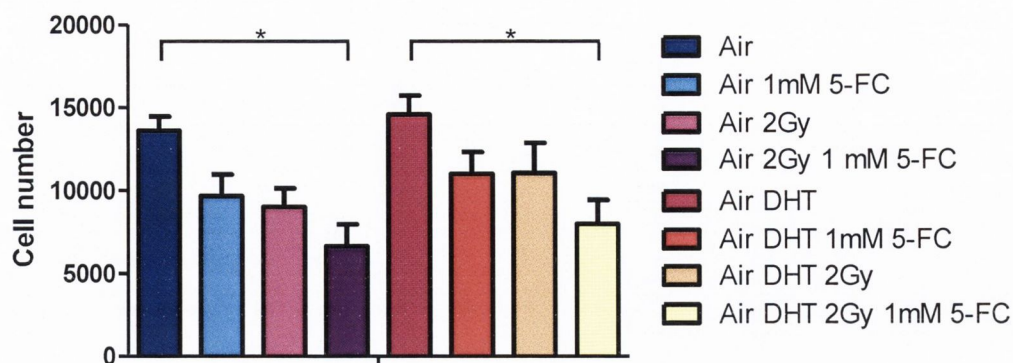


Figure 7.14: pPPE-CD transfected 22Rv1 cell number after treatment in air with radiation and/or 5-FC. Values represent the mean $\pm$ standard error of the mean. *, $p < 0.05$

For cells grown under hypoxic conditions (0.5% oxygen), both hypoxia alone and hypoxia in combination with DHT treatment resulted in a significant decrease in the number of pPPE-CD transfectants with 1 mM 5-FC treatment, which was not the case for untransfected cells ($p < 0.001$, Figure 7.15). The number of pCMV-CD transfectants also decreased significantly with 5-FC treatment ($p < 0.01$). When hypoxia treatment was combined with radiation, 5-FC treatment also resulted in a significant decrease in cells expressing pPPE-CD but not untransfected cells. This was the case both with and without DHT stimulation ($p < 0.01$, Figure 7.16).

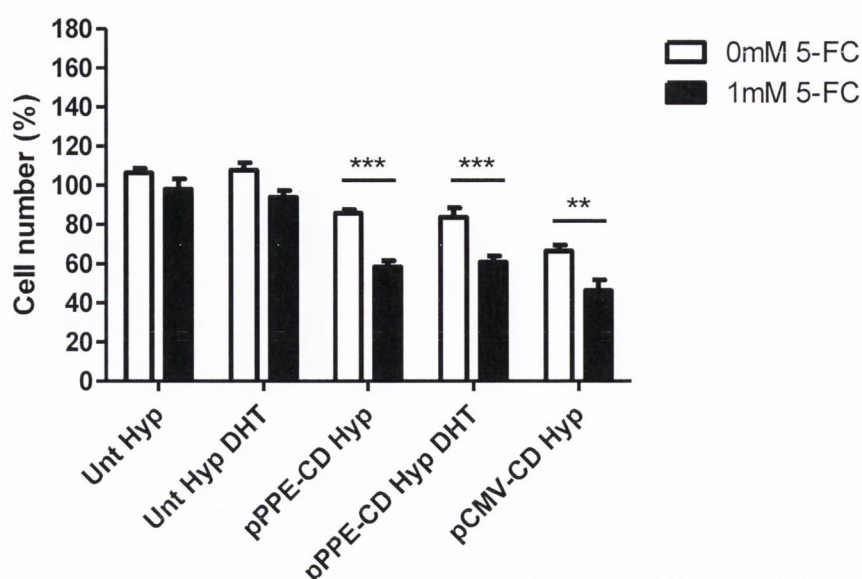


Figure 7.15: 22Rv1 cell count after treatment in hypoxia with and without DHT. Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected; ***, $p < 0.001$; **, $p < 0.01$.

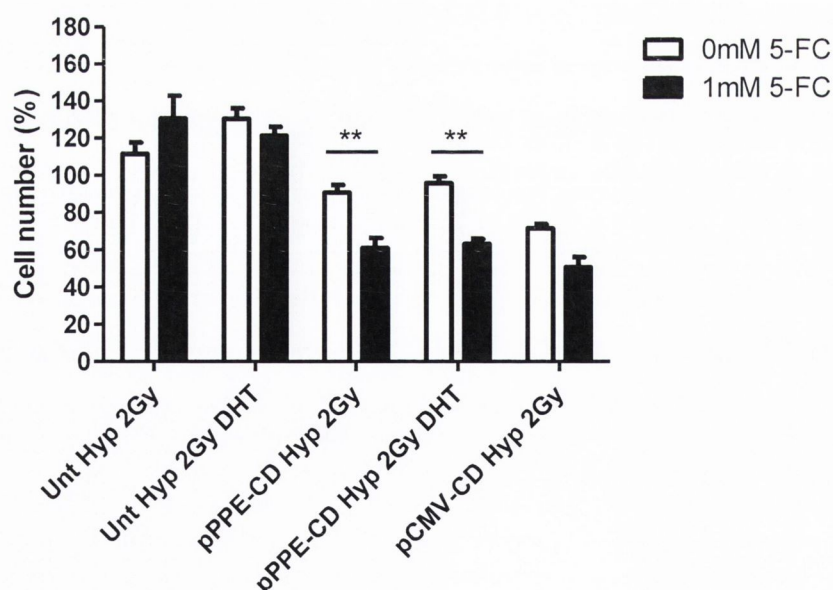


Figure 7.16: 22Rv1 cell count after treatment in hypoxia with radiation and with and without DHT. Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected; **, $p < 0.01$.

22Rv1 spheroids

A number of parameters were analysed to assess the efficacy of pPPE-CD/5-FC treatment in the spheroid model of 22Rv1. There was no significant difference in spheroid number with 5-FC treatment for any of the cell types (untransfected, pPPE-CD or pCMV-CD transfected) under the various growth conditions. Spheroid size was also analysed. The results were expressed as a percentage of the untransfected control untreated with 5-FC. A significant decrease in the average spheroid size was observed for pPPE-CD spheroids grown in air and treated with 1mM 5-FC compared to the equivalent samples without 5-FC ($p < 0.001$, Figure 7.17). 5-FC treatment under aerobic conditions resulted in a significant decrease in pCMV-CD spheroid size also ($p < 0.05$) but this was not the case for untransfected cells. For spheroids grown under aerobic conditions (21% oxygen) with 2 Gy radiation treatment, there was a significant decrease in average size for radiation alone ($p < 0.05$) and the degree of significance increased for 2 Gy and DHT treatment combined ($p < 0.01$, Figure 7.18). Under hypoxic conditions (0.5% oxygen), there was a significant decrease in the average spheroid size for pPPE-CD spheroids treated with 5-FC ($p < 0.5$, Figure 7.19). When hypoxia treatment was combined with radiation, the change in spheroid size in response to 5-FC no longer reached significance (Figure 7.20).

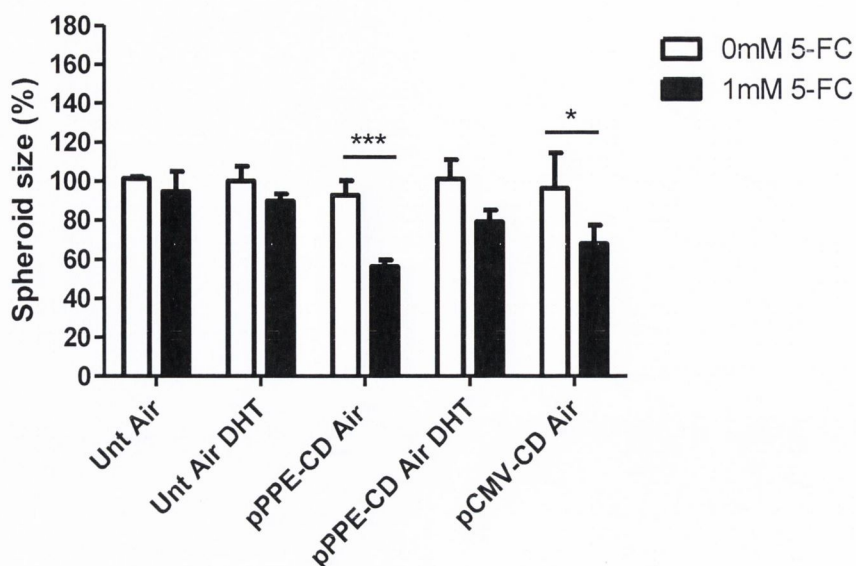


Figure 7.17: 22Rv1 spheroid size after treatment in air with and without DHT. Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected; ***, $p < 0.001$; *, $p < 0.5$.

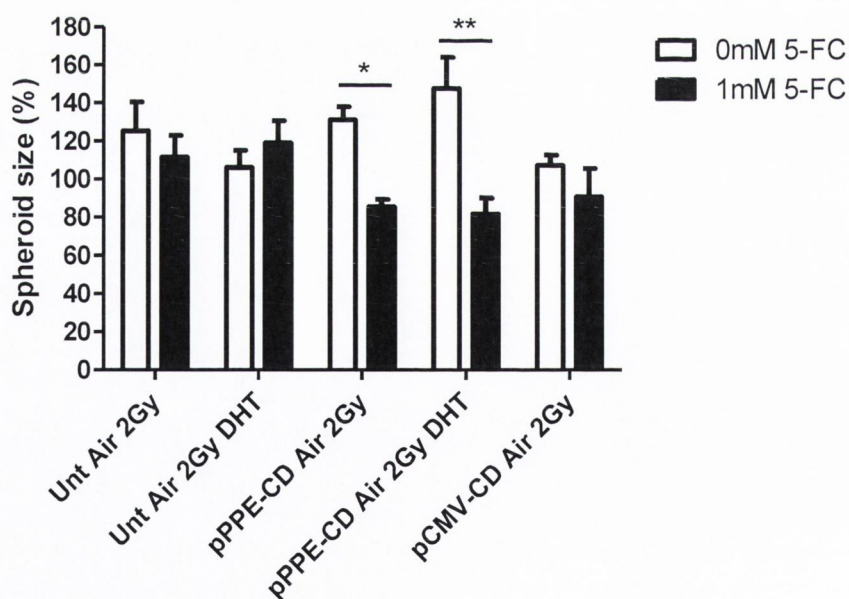


Figure 7.18: 22Rv1 spheroid size after treatment in air with radiation and with and without DHT. Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected; **, $p < 0.01$; *, $p < 0.5$.

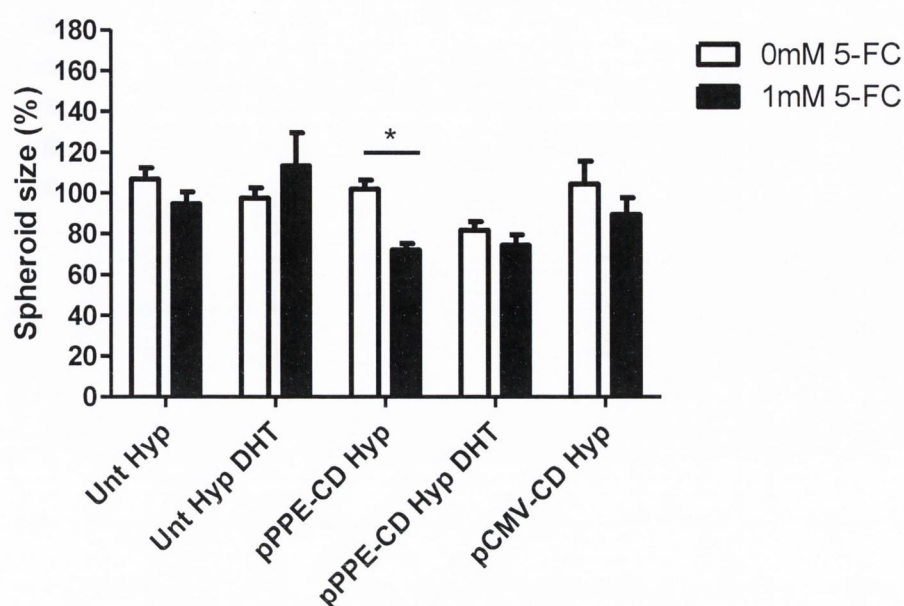


Figure 7.19: 22Rv1 spheroid size after treatment in hypoxia with and without DHT. Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected; *, $p < 0.5$.

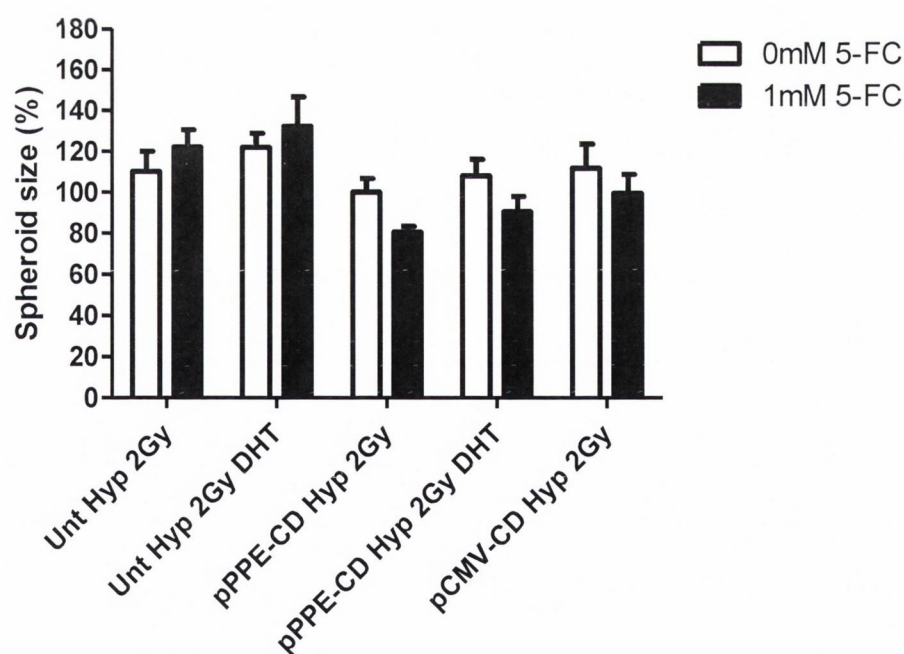


Figure 7.20: 22Rv1 spheroid size after treatment in hypoxia with radiation and with and without DHT. Results are expressed as a proportion of untransfected untreated 22Rv1. Values represent the mean $\pm$ standard error of the mean. Unt, untransfected.

When the raw values for spheroid size are compared between those cells treated under aerobic conditions with DHT and with and without radiation, the combined effect of 5-FC and radiation can be assessed using a one-way ANOVA with Bonferroni multiple comparison test. As was the case with adherent pPPE-CD-transfected 22Rv1 cells analysed previously (Figure 7.14), neither 2 Gy radiation treatment nor 1 mM 5-FC were sufficient alone to result in a significant decrease in spheroid size compared to untreated spheroids (Figure 7.21). However when the two treatments were combined the difference was highly significant ($p < 0.001$).

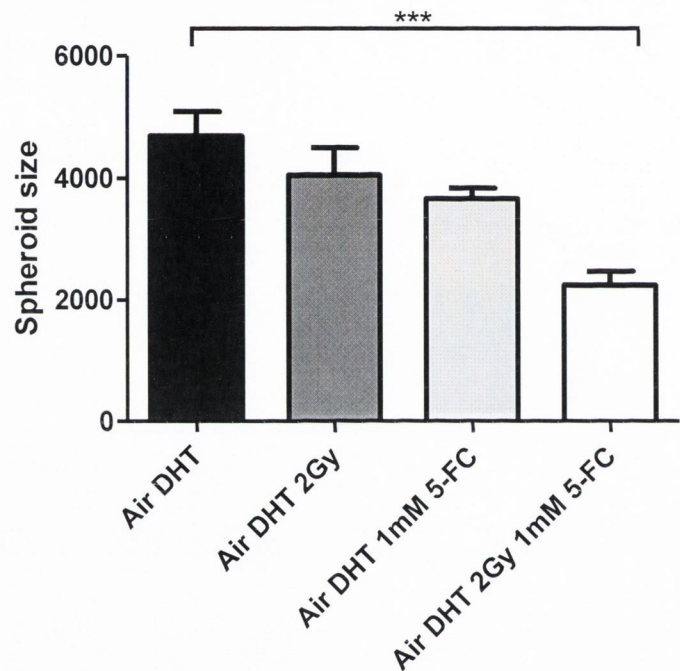


Figure 7.21: pPPE-CD transfected 22Rv1 spheroid size after treatment in air with radiation and/or 5-FC. Values represent the mean $\pm$ standard error of the mean. ***, $p < 0.001$.

7.3 Discussion

This research describes a novel technique for the formation of tumour spheroids. It has long been believed that growing tumour cell lines as spheroids is an improved model that is closer to the conditions *in vivo* compared to their growth as adherent monolayers. Despite this, their use has not become widespread and possible barriers may include the difficult and time-consuming nature of some of the current techniques and the need for specialist equipment. Also, if use of spheroids is to become standard practice for high-throughput screening, the method of formation needs to have ease of use and scalability. This technique for spheroid formation goes some way to addressing these issues. Once optimised, spheroid formation was as straightforward and simple as growing cells as a monolayer. Some techniques had to be adapted as the cells were in suspension in the media, meaning the media could not be removed and replaced as it would be with monolayer cell culture. Instead, drug-containing media was layered on top of the existing media to allow diffusion. This same method was used for the control 22Rv1 cells grown with the typical 2D cell culture.

It was important to establish that the cells had indeed formed spheroids and not aggregates (loosely packed bundles) of cells which lack cell-cell and cell-matrix interactions and will not have the pathophysiological gradients found in genuine spheroids.²⁴⁵ Unfortunately there are inconsistencies in the literature in the nomenclature used, with spheroids also being called spheres, organoids and tumoroids, but more importantly so-called spheroids actually being aggregates. Aggregates easily fall apart and cannot be manipulated. The 22Rv1 spheroids developed in this chapter remained intact when transferred from well to well and did not break apart easily.

As spheroids increase in size they develop a core of necrotic cells, something which models the situation *in vivo*, in which the peripheral cells of a tumour have access to nutrients and oxygen, whereas those further from a blood supply may die. In initial confocal microscopy, the stain propidium iodide, a membrane impermeant DNA dye, was restricted to the inner cells of the spheroid, suggesting they were necrotic. The nuclear dye Hoechst, showed the cells were healthy on the periphery of the spheroids and further confirmed the densely packed structure of the spheroids.

At sizes of 200 – 500 μm , chemical gradients (in oxygen or nutrients for example) will occur in the spheroids.²⁴⁵ In the case of the 22Rv1 spheroids developed during the course of this research, their size suggests they would be large enough for chemical gradients to develop. Future work will involve using hypoxia markers, such as pimonidazole, to test this hypothesis. If these spheroids have gradients of oxygen concentration, it may not be necessary to use the hypoxia chamber and the model would be closer to *in vivo* conditions where levels of hypoxia vary in different parts of the tumour.²⁴⁹ Khaitan *et al.* described spheroids as being “ideally suited for developing novel therapies that...exploit the endogenous oxidative stress in tumours.”²⁴⁹

The cells were tracked from the point of seeding in HC media, to try to elucidate the mechanism of spheroid formation. Between time of seeding and 24 hours later the number of individual cells had greatly decreased and the wells contained spheres of cells packed together suggesting that the cells travel through the media and aggregate in initial spheroid formation. Over the following days the spheroids became larger and more rounded suggesting cell division may also play a role. It would be informative to image the wells at shorter and more frequent intervals between seeding and Day 1 to gain more information on the movement of cells through the HC media. It may also be useful to assay secreted proteins in the media to help elucidate molecular cell signalling mechanisms behind spheroid formation and maintenance.

The experiments using high-throughput technology for automatic liquid handling were preliminary but were encouraging. HC media seems to be an improvement on normal DMEM media when it comes to the issue of cells sinking in the source cell suspension, resulting in decreased cell numbers over time during automatic liquid dispensing. Nanowell slides were used to test the capacity for spheroid formation on a nanolitre scale, which would be conducive to high throughput drug screening as it would allow large scale testing using very small amounts of the drug of interest. HC media and 22Rv1 spheroids showed potential in this regard and future work could involve testing established drugs in this setting to further develop this technique.

Previous attempts to increase PSA expression in 22Rv1 cells through the overexpression of AR were not successful (Chapter 6). Before using the 22Rv1

spheroids as a model in which to test the pPPE-CD/5-FC system, levels of secreted PSA were measured to determine whether the growth of the cells in 3D cell culture had an effect on PSA expression. The cells were seeded and following spheroid formation, DHT was added to half of the wells, with ethanol added to the other half. Adherent cells were used as a control, with one set grown in HC media from the point of DHT addition onwards, to ensure any difference in PSA levels was due to growth as spheroids, not the media itself. Secreted PSA levels were measured after 3 days of DHT treatment and normalised to cell number to control for any disparity in cell proliferation rates between the 2D and 3D cultures. In the absence of DHT, the baseline level of PSA was significantly higher for the spheroids compared to the adherent cells ($p < 0.05$) and this increase was not observed for the adherent cells grown with HC media. With the addition of 1000 nM DHT, the PSA levels approximately doubled for both adherent cells and spheroids. Once again, PSA secretion was significantly higher for the spheroids ($p < 0.05$). The level of PSA for the spheroids with no DHT treatment was as high as the level of PSA for adherent cells with DHT treatment. This was an encouraging result, suggesting not only that 22Rv1 spheroids may be an improved model in which to test pPPE-CD but also that they may better reflect the PSA levels of primary tumour cell *in vivo* and be a good *in vitro* system in general.

The media used, both for spheroids and adherent cells, contained normal FBS, not charcoal-dextran stripped FBS as the HC media was a prototype and was still under development. Although this may have affected the levels of PSA produced by the cells, the adherent cells were grown in complete DMEM media as well as the spheroids, with the only difference being the additional additive in the HC media. Therefore the significantly higher levels of PSA secreted by the spheroids should not be a result of any difference in hormone concentration between the normal and HC media. To further confirm this, future experiments will use HC media developed using CD-FBS.

The exact reasons for the higher PSA protein levels detected for spheroid samples are not clear. Differences in gene expression are quite likely, considering this has been demonstrated to be the case for a range of tumour types, including prostate, when spheroids were compared to the equivalent adherent cells.^{248, 250-252} It would

be informative to examine genome-wide mRNA expression levels and their differences between 22Rv1 spheroids and adherent cells with and without DHT stimulation. This could be achieved through microarray transcription analysis which would involve a hybridisation-based pairwise assay of relative transcript expression levels between the two samples. Genome-wide expression arrays exist, which could give information about the expression levels of ~20,000 transcripts. Alternatively, genome-wide mRNA expression levels could be assayed through next-generation sequencing using RNAseq, which has the benefit over microarray-based methods that novel transcripts or those not included in the microarray design can be directly assayed.²⁵⁷ These experiments would be informative as they would give detailed information on the genes up- and downregulated in spheroids compared to adherent 22Rv1, as well as in response to DHT treatment. Gene ontology (GO) and pathway (KEGG) analysis of overrepresented categories in the differentially expressed gene group would provide useful information and would help elucidate possible reasons for higher PSA expression levels in spheroids.

Transfection of 22Rv1 cells with a GFP-expressing vector, followed by growth in HC media, demonstrated that transfection of the cells did not affect their ability to form spheroids. The cells were tracked for up to 7 days after spheroid formation to ensure the GFP expression was still strong as this indicated that pPPE-CD expression would last sufficient time to allow growth of the spheroids after transfection, followed by treatment of the cells with DHT/hypoxia/radiation, and then 5-FC.

The activity of the pPPE-CD/5-FC system was tested in spheroids alongside adherent cells using Hoechst staining and high-content screening to assess the change in spheroid size/cell number with treatment. The results for the adherent cells showed the efficacy of this treatment strategy again, following on from the MTT and clonogenics results from Chapters 3 and 4. The number of cells was normalised within each replicate and compared between equivalent samples with and without 5-FC. For pPPE-CD cells grown in air, treated with DHT or 2 Gy radiation prior to 5-FC, the decrease in cell number did not reach significance compared to the samples without 5-FC. However, when DHT and radiation treatment were combined, the difference was significant ($p < 0.01$), serving as

evidence for the benefit of a combined treatment strategy. pPPE-CD-22Rv1 grown in hypoxia (0.5% oxygen) alone, as well as in hypoxia with DHT, showed a significant decrease in cell number with 5-FC treatment ($p < 0.001$). Growth in 0.5% oxygen activates pPPE-CD activity, resulting in 5-FC to 5-FU conversion and consequent cell toxicity. This decrease in cell number was also observed for cells grown in hypoxia with or without DHT that were irradiated ($p < 0.01$). These results are further confirmation of the increase efficacy of pPPE-CD under hypoxic conditions and the potential benefits of a combined treatment strategy involving radiation and the pPPE-CD/5-FC system.

For the spheroid analysis, two parameters were measured, spheroid number and spheroid size, using the same Hoechst staining and high-content screening protocol as was used for the adherent cells. The pPPE-CD transfected spheroids showed no difference in number between 5-FC treated and untreated samples for any of the growth conditions. This was not surprising as the spheroids were allowed to form before their growth under various conditions (hypoxia, DHT) followed by 5-FC treatment. There were differences in spheroid size, however, with pPPE-CD/5-FC toxicity resulting in a significant decrease in size for spheroids grown under both aerobic (21% oxygen) and hypoxic (0.5%) conditions. Interestingly, the difference did not reach significance if the spheroids were grown under aerobic or hypoxic conditions with DHT. When PSA levels were measured, the baseline expression for the cells grown in spheroids was as high as expression for the adherent cells treated with 1000 nM DHT. Therefore, it is not surprising that pPPE-CD was activated without DHT stimulation in this model. It is unclear why stimulation with DHT would not further increase the activity of pPPE-CD. Treatment of the aerobic spheroids with 2Gy radiation with and without DHT, resulted in a significant decrease in spheroid size for the 5-FC treated samples. These results indicate that pPPE-CD/5-FC treatment can result in cell toxicity in a spheroid model and therefore shows potential in a system closer to *in vivo* conditions than cells grown in 2D cell culture.

There is debate over the most suitable and informative endpoint to measure after spheroid treatment and a range of different techniques have been used. An automated approach has definite advantages, especially for large scale drug

screening. Changes in spheroid volume and growth delay after treatment are often measured and can be achieved using software capable of such image analysis. Automated screening was used during this project and measurement of spheroid size served as a straightforward indicator of degree of toxicity. Other groups have used the MTT assay, which was shown to be more sensitive than the lactate dehydrogenase (LDH) assay, cell growth or DNA assay.²⁵⁸ However this has mainly been tested in the context of smaller spheroids (less than 200 μm).²⁴⁶ A range of different assays commonly used in the study of monolayer cultures were compared for their suitability in spheroid analysis using the colon carcinoma lines HT29 and HCT-116.²⁵⁵ It was found that the acid phosphatase assay is suitable as it does not require spheroid dissociation and it is linear and sensitive across different spheroid sizes. Future work could involve the use of this assay to further test the activity of pPPE-CD in prostate cancer spheroids.

5-FU, the toxic form of the prodrug used in this research, is a chemotherapeutic drug whose activity has been tested in spheroid models of cancer and compared to the equivalent 2D models. As discussed in section 7.1, the efficacy of 5-FU is reduced in 3D cell culture, the likely reason being the lower proliferation rates.²⁴⁷ It was encouraging, therefore, to find a toxic effect with pPPE-CD/5-FC treatment in the spheroid model of prostate cancer. It would be informative to compare directly the effect of 5-FU between 22Rv1 cells in 2D and 3D culture using an assay such as the acid phosphatase assay. This would help in understanding the processes occurring in 22Rv1-pPPE-CD spheroids treated with 5-FC – it is possible that the levels of activation of the PSA promoter are higher, leading to greater CD expression, but that the subsequent toxicity of 5-FU is lower.

This chapter discusses the development of a new technique for tumour spheroid formation and the results obtained indicate its potential as a model system. There are numerous experiments that could now be carried out to further develop and test these spheroids as well as the possibility of growing other prostate tumour cell lines as spheroids. It would be valuable to build up a panel of prostate tumour spheroids with which to test pPPE-CD/5-FC efficacy as well as having potential for wider use in the study of prostate tumour growth and progression and for use in drug development.

Chapter 8

Discussion and conclusions

Chapter 8 Discussion and conclusions

Prostate cancer is the most frequently diagnosed form of cancer for Irish men with a 2007-2009 diagnosis rate of 140.1 per 100,000 per year and a mortality rate of 16.5 per 100,000 per year for the same period.⁸ Although the current treatment strategies for localised forms of prostate cancer, radical radiation therapy and retropubic radical prostatectomy, are often effective, a subset of men experience treatment failure and recurrence of the disease and once this happens, the follow-up treatment options are non-curative. Therefore any improvement in the available therapies for local or advanced prostate cancer would be beneficial.

This project explored the potential of a GDEPT system for the treatment of prostate cancer and as an adjuvant to radiation therapy. The prostate is a good candidate for this form of therapy as it can be easily accessed for administration of drugs, which can occur in an outpatient setting, reducing costs. Previous research in the Prostate Molecular Oncology Research Group produced the vector pPPE-CD, which contains the PSA promoter and enhancer upstream of the CD gene and is combined with the prodrug 5-FC to convert it to its toxic form 5-FU. 5-FU has the advantage of already being an established chemotherapeutic agent, particularly in the treatment of colorectal cancer. However, systemic administration of the drug can result in serious side-effects, including neurological damage, as is the case with all types of chemotherapy.²⁵⁹ Recent research has led to an improved understanding of this clinical problem: short-term systemic administration of clinically relevant doses of 5-FU in mouse models results in damage to central nervous system progenitor cells and oligodendrocytes and subsequently leads to chronic degeneration of the central nervous system.²⁵⁹

One possible way of preventing these undesirable side-effects is to use a GDEPT system to target the chemotherapeutic drug. By administering the drug in the form of a low toxicity prodrug and controlling expression of the enzyme that converts it to its toxic form so that it is specific to the organ of interest, high levels of cell kill can be achieved without the concurrent side-effects. It was demonstrated by Dr. Ruth Foley that CD expression from pPPE-CD is upregulated by treatment with DHT. Vector expression was shown to be specific as 5-FC treatment resulted in cell

kill in transfected 22Rv1 and LNCaP cells but not in AR-negative PC3 or the colon cancer cell line HCT116.

8.1 Improved efficacy of pPPE-CD under hypoxic conditions

Research suggests that there is cross-talk between gene regulation by HIF-1 and AR in LNCaP.^{190, 211, 212} DHT stimulation has been shown to enhance hypoxia-regulated expression of the gene GLUT-1 and synergistically PSA expression increases when hypoxia is combined with DHT treatment, which is of particular relevance to this project.¹⁹⁰ The importance of ARE III in the PSA promoter has been demonstrated as well as the identification of a hypoxia response element which allows for PSA stimulation by hypoxia alone.¹⁹⁰ In light of this evidence, the activity of pPPE-CD was tested under hypoxic conditions to determine if this affected CD expression levels.

This study of pPPE-CD under hypoxic conditions (0.5% oxygen) yielded results that underscore the potential for exploitation of the AR/HIF-1 cross-talk in GDEPT for prostate cancer. The presence of oxygen is necessary for successful radiation therapy and hypoxia has been implicated in treatment resistance. Therefore, targeting hypoxic cells within the prostate tumour using a GDEPT system, alongside radiation treatment, could kill those cells most likely to be resistant and decrease the risk of recurrence. Testing of pPPE-CD under hypoxic conditions in 22Rv1 and LNCaP cells indicated that CD protein levels were higher when hypoxia and DHT stimulation were combined than for DHT treatment under aerobic conditions and that hypoxia alone was capable of activating CD expression in 22Rv1. This was an encouraging result and evidence that the AR/HIF-1 cross-talk did indeed influence CD levels. Results of MTT assays to measure the toxicity of pPPE-CD/5-FC, showed that the difference in cell viability between untransfected and pPPE-CD-22Rv1 with DHT was significant for aerobic samples ($p < 0.05$) but the degree of significance was greater for hypoxic samples ($p < 0.001$). The results of clonogenic assays testing this effect were less clear with possible reasons for this discussed in section 5.3.

In Chapter 7, this efficacy of pPPE-CD was further tested in adherent 22Rv1 and analysed by Hoechst staining and high-content screening. A significant decrease in cell number was not observed for 5-FC treated pPPE-CD-22Rv1 grown in air with

or without DHT compared to the equivalent samples without 5-FC. However, when hypoxia (0.5% oxygen) was used with or without DHT, the difference was highly significant ($p < 0.001$). These results strongly suggest that the PSA promoter/enhancer in pPPE-CD can be controlled using hypoxia as well as DHT stimulation and confirm the hypothesis that the cross-talk between HIF-1 and AR in the control of gene expression extends to the PSA promoter/enhancer used in the pPPE-CD/5-FC system.

A comparison conducted by Zhong *et al.* of HIF-1 α expression levels between normal and malignant tissue for a range of tumour types found that for prostate samples, it was absent in normal tissue but present in most tumour samples.²¹⁷ Immunohistochemical staining of prostate tissue samples found significantly higher levels of HIF-1 α , VEGF and CD34 (a marker of microvessel density) in tumour samples compared to BPH, with degree of expression correlating with tumour grade.²⁶⁰ The research presented in this thesis has demonstrated an additional level of control of pPPE-CD activity which would be advantageous. As mentioned, PSA promoter expression has been shown to be highly prostate-specific. Being hypoxia-inducible makes pPPE-CD tumour selective and ensures the highest levels of CD expression are within the hypoxic, treatment-resistant tumour cells.

8.2 A combined treatment strategy of pPPE-CD/5-FC and radiation therapy

There have been significant technological advances in the field of radiation therapy which have led to improvements in delivery and imaging. The traditional two-dimensional method of administering radiation beams has, for the most part, been replaced with 3D-CRT which allows for greater precision of beam targeting.^{60, 65} This means that higher doses can be administered, increasing the chance of tumour eradication. IMRT and brachytherapy represent other advances in this area. Dose escalation has improved tumour control and recurrence rates but there can be complications. For example, for patients treated with 3D-CRT, the risk of late rectal radiation toxicity at 5 years has been shown to be 15.6% for grade 2 and 7% for grade 3 toxicity.²⁶¹ It is also thought that there could be subpopulations of clonogenic tumour cells that are resistant to radiation treatment, which may increase the chance of local recurrence or metastasis in a proportion of the patients.⁵⁹ Alternative ways to improve the efficacy of radiation therapy would be beneficial.

A combined treatment strategy for prostate cancer involving radiation therapy and pPPE-CD/5-FC would appear to have a number of potential benefits. Research in Chapter 4 established that pPPE-CD activity is increased when the vector is stimulated with DHT under hypoxic conditions (0.5% oxygen). The presence of regions of hypoxia is an important factor in determining the efficacy of radiation treatment.⁵³ In the absence of oxygen, the free radicals formed on the DNA as a result of irradiation are reduced by hydrogen instead of oxidized, which allows for repair of the DNA.⁵² In general, the presence of hypoxia indicates a poor prognosis for prostate cancer patients; it is associated with tumour aggressiveness and metastasis as the transcription factor HIF-1 upregulates genes involved in angiogenesis and glycolysis.^{43, 50} It is clear that hypoxia plays a significant role in prostate cancer outcome so targeting hypoxic cells in particular could improve the impact of radiation treatment and ultimately reduce the risk of relapse.

Combining pPPE-CD/5-FC with radiation therapy could also increase its efficacy as 5-FU has been identified as a radiosensitizer, as well as being cytotoxic and therefore could enhance the amount of cell death induced by ionizing radiation. For example, the use of 5-FU combined with radiotherapy is standard in the treatment of gastrointestinal tumours.²⁶² Investigation has been carried out to assess the potential benefit of using the CD/5-FC system along with radiotherapy. Recently, Liu *et al.* looked at the effect of this combined treatment in mice bearing xenografts of the human cervical epithelial cell line HeLa. It was shown that in the case of both single (12.5 Gy) and fractionated (3 Gy $\times$ 5 days) radiotherapy, the efficacy of the treatment was improved with minimal side effects. The tumour growth doubling time increased by 2.4 times and 2.5 times for single and fractionated treatments respectively, compared to radiotherapy alone.¹⁸⁰

In the case of prostate cancer, Anello *et al.* assessed the outcome of using the CD/5-FU system in the mouse prostate cancer cell line RM-1, combined with low doses of radiation. This resulted in 2 logs of cell kill. Highest levels of cell killing were observed when 5-FC administration took place 24 hours before radiotherapy.¹¹² Similar research was carried out by Kato *et al.* and involved mice bearing xenografts of the prostate cancer cell line LNCaP expressing CD. The effects of administration of 5-FC were compared with various doses of 5-FU. The antitumour

effects of CD/5-FC therapy were significant and, unlike systemic use of 5-FU, did not cause weight loss in the mouse models. This inhibition of tumour growth was improved when partnered with 1 Gy irradiation.²⁶³

The mechanism controlling radiosensitivity following drug treatment has not been fully elucidated and requires further study. Despite this, if pPPE-CD/5-FC is combined with radiation treatment of localized prostate cancer it may allow for increased levels of cell kill without needing to increase radiation dose levels, thereby reducing the chance of toxic side effects.

In Chapter 5, pPPE-CD activity was tested in combination with radiation treatment to investigate the potential of this GDEPT system as an adjuvant. This permitted the assessment of the efficacy of a combined treatment strategy when compared to GDEPT or radiation treatment alone. Experiments were undertaken to determine if radiation would have any influence on the efficacy of pPPE-CD/5-FC. Western blotting demonstrated that CD expression is upregulated in 22Rv1 and LNCaP pPPE-CD transfectants treated with 2 Gy radiation and DHT compared to DHT alone. Further examination was carried out to determine if radiation treatment increased the toxicity of pPPE-CD/5-FC. Cell viability assays showed that the decrease in pPPE-CD transfectant cells treated with 5-FC compared to untransfected control cells was significant for DHT treatment in air ($p < 0.05$) but this was more pronounced if 2 Gy radiation was also given ($p < 0.01$). Further study involved clonogenic assays and high-content screening (Chapter 7) which also indicated an increase in the toxicity of pPPE-CD/5-FC with 2 Gy radiation treatment in 22Rv1 cells. These results are promising as they suggest a combined strategy could improve the efficacy of either radiation therapy or pPPE-CD/5-FC given alone. It appears that there is a synergistic relationship between the two treatments, with radiation improving the efficacy of the pPPE-CD/5-FC as well as the potential increase in radiosensitivity resulting from 5-FU. These findings imply that radiation could be used to further control expression of CD in the tumour, perhaps to target particular parts of the tumour.

8.3 Suitable controls for qRT-PCR analysis of hypoxic prostate cell samples

Prostate cancer cell lines (LNCaP, 22Rv1, PC3 and DU145) and normal prostate cell lines (PWR1E and RWPE1) were cultured under aerobic (21% oxygen) and

hypoxic (0.5%) conditions for either 24 or 48 hours. The performance of 16 endogenous control genes was assessed using the statistical programme Normfinder and by measuring the coefficient of variation (C_tC_v). Hypoxia resulted in marked changes in expression levels of several genes including *PGKI*, *IPO8* and *B2M*. There was also disparity between cell lines, although *UBC* was stably expressed across all lines tested. *ACTB* was the best performing gene for 22Rv1 and LNCaP and was selected for use in future experiments in this project comparing aerobic (21% oxygen) and hypoxic (0.5%) samples. The impact of the endogenous control gene on qRT-PCR was explored by quantifying expression of target genes *HIF-1A* and *GSTP1* in cell lines and tumour tissues, respectively. The use of *PGKI* as an endogenous control was shown to significantly impact on the interpretation of *HIF-1A* expression, when compared to stably expressed HKGs *ACTB* and *UBC*. Good endogenous controls in prostate cell lines were not necessarily good controls in archived prostate tissue specimens, in which *ACTB* was found to be unsuitable for assessment of *GSTP1* expression levels.

This research highlights the importance of testing potential control genes before normalisation of qRT-PCR results. Variation in their expression makes some genes unsuitable for experiments involving hypoxia. *UBC* performed well in all cell lines tested but a ‘universal control’ does not exist and re-testing is required before introducing any new cell line or treatment condition.

8.3.1 Cell lines

As a first line model for prostate cancer research, cell lines are a useful tool. They are readily available and once purchased, stocks can be maintained reasonably cheaply and easily. There is a range of different prostate cancer cell lines available that are representative of different stages of disease progression. The original three cell line used were LNCaP, PC3 and DU145 and they are still the most popular today.²⁶⁴ Of the three, LNCaP is the only one which is androgen sensitive and expresses AR and PSA. It was derived from biopsy of a lymph node metastatic lesion of a 50-year old man.²⁶⁵ DU145 was the first prostate cancer cell line to be established and came from epithelial cells that originated from the brain metastases of a 69-year old man.²⁶⁶ PC3 was derived from a lumbar vertebral metastasis of a 62-year old.²⁶⁷

Cell lines derived from primary tumours are less common, the most well established being 22Rv1. 22Rv1 are derived from the xenograft line CWR22R which was formed from primary prostate tumour tissue from a patient who also had bone tissue metastases.²¹⁸ Together with LNCaP, 22Rv1 cells were used during the course of this research. Although 22Rv1 cells express AR and secrete PSA, they do not mimic the high PSA levels expressed by localised prostate tumours *in vivo*. One aim of his research project was to stably transfect 22Rv1 so that they would overexpress AR in an attempt to make an improved model in which to test pPPE-CD. The transfection was successful but this did not result in the higher levels of PSA expression expected. Even when activation of PSA promoter was quite low (as demonstrated with PSA EIAs and qRT-PCR), CD expression was still evident and followed the same pattern of expression observed in the parent cell line. CD protein levels were higher under hypoxic (0.5% oxygen) compared to aerobic (21%) conditions and were also higher when cells were treated with 2 Gy of radiation and DHT under aerobic conditions compared to DHT alone. Although the original aim was to increase PSA expression in 22Rv1, this model may in fact be a good mimic for stages of the disease with low PSA levels, for example, initial stages of androgen deprivation therapy. It is encouraging that despite the low levels of PSA upregulation, CD expression in transfected 22Rv1-ARhi cells was still found to be strong.

8.3.2 Spheroids

22Rv1 spheroids were developed as another attempt to improve the standard *in vitro* models of primary prostate cancer. This involved a new form of media, Happy Cell (HC), produced by the Trinity College start-up company Biocroí which was proposed to allow spheroid formation. This research was the first thorough investigation of the potential of HC media and was successful in demonstrating spheroid formation using this tool.

pPPE-CD/5-FC showed potential in this model, with a significant decrease in the size of pPPE-CD transfected spheroids grown under aerobic (21% oxygen) or hypoxic (0.5%) conditions and treated with 5-FC compared to the equivalent spheroids without 5-FC. This difference was not found for mock transfected spheroids. These results are further evidence for the potential of this kind of GDEPT

strategy, this time demonstrated in a model of prostate cancer which more closely reflects the conditions *in vivo*.

HC media is likely to have commercial potential and be an improved *in vitro* system in which to investigate tumour behaviour and test potential drugs or therapies. Growing cells as an adherent monolayer causes the loss of certain cell characteristics and an exaggeration of others, such as an artificially high proliferation rate.²⁴⁸ This has a significant impact on the types of drugs which are effective in this model and does not necessarily select for drugs which will show potential *in vivo*. Testing new drugs in tumour spheroids would likely rule out many drugs at this early stage and reduce the need for costly and time-consuming animal xenograft models.

8.4 Possible improvements of the PSA promoter

This research involved a vector whose activity was controlled by the PSA promoter/enhancer and it was demonstrated to be androgen, hypoxia and radiation-inducible. It may be beneficial to modify the PSA promoter to improve activity, while still maintaining tissue-specificity as well as activity in response to the various treatment conditions mentioned. Tandem duplication of the PSA enhancer has been shown to increase promoter activity resulting in a 50-fold increase in luciferase expression in PSA-positive cells, approaching the level of CMV-controlled expression.¹⁶⁹ Creation of an adenoviral vector in which this promoter controlled the enzyme nitroreductase resulted in prostate-cell specific sensitivity to the prodrug CB1954.

Wu *et al.* tried multiple strategies to improve the activity of the PSA enhancer. Compared to the unmodified construct, duplication of the enhancer core (PSE-BC), insertion of four tandem repeats of ARE I (PSE-BA) and a combination of the two (PSE-BAC) resulted in upregulation by 18.9, 7.3 and 9.4 fold respectively.²⁶⁸ Biochemical analysis demonstrated that this correlated with increased recruitment of AR and its co-regulators. The chimeras remained prostate-specific and this was maintained when an adenoviral construct controlled by PSE-BC was administered to mice with LAPC-9 human prostate cancer xenografts.

An alternative to direct modification of the PSA promoter or enhancer is two-step transcriptional amplification (TSTA) system. This adds an extra step to the process, so that the tissue specific promoter, such as PSA, controls expression of a powerful transcriptional activator which regulates a second expression plasmid containing the therapeutic gene.²⁶⁹ The activator has frequently been GAL4–VP16 which is a fusion protein composed from the DNA binding domain of the yeast gene regulator GAL4 and the herpes simplex virus activation domain VP16.^{269, 270} Optimisation experiments were carried out using two different sequences from the PSA promoter/enhancer and one, two, or five copies of the GAL4 binding site, controlling expression of luciferase.²⁶⁹ The TSTA system was compared to a one-step system in which the PSA promoter/enhancer system directly controlled luciferase expression and was found to increase activity and androgen responsiveness and was highest for the plasmid containing 5 GAL4 binding sites. An 800 fold enhancement in activity was achieved for the TSTA vs. one-step system. Further investigation using a TSTA adenovirus vector to express HSV-tk in mouse models demonstrated that expression was strong and tumour-specific and did not result in the liver toxicity observed when a constitutive promoter was used.²⁷¹

8.5 Possible improvements in CD efficiency

Various factors can reduce the sensitivity of mammalian cells to 5-FC/CD treatment compared to direct 5-FU administration. 5-FC can only enter the cells by passive diffusion, and once formed, 5-FU may be degraded to non-toxic compounds by dihydropyrimidine dehydrogenase and the de novo synthesis of UMP dilutes the amount of 5-FUMP.²⁷² The efficiency of CD conversion of 5-FC to a toxic drug can be improved using a double GDEPT system in which uracil phosphoribosyltransferase (UPRT) is also expressed, which converts 5-FU to 5-FdUMP which in turn can inhibit thymidylate synthase to prevent DNA synthesis. Initial experiments involved combining the *E. coli upp* gene encoding UPRT with the gene for CD in an *E. coli*-animal cell shuttle plasmid which was tested in *E. coli* strains developed to mimic 5-FC metabolism in mammalian cells.²⁷² Sensitivity to 5-FC increased significantly compared to plasmids containing the CD gene alone.

Two alternative methods have been developed to combine CD and UPRT: the use of two separate vectors or one vector expressing a fusion gene CD-UPRT. Both

strategies have been shown to be effective. In the prostate cancer cell line DU145, a treatment strategy involving two adenoviral vectors expressing *E. coli* CD and UPRT was compared to the CD-expressing vector alone and was shown to increase sensitivity to 5-FC.²⁷³ This effect was also observed *in vivo* in the treatment of DU145 tumours grown in nude mice. A fusion gene-expressing vector was used to treat mice with intraprostatic androgen-refractory prostate (RM1) tumours and lung metastases.²⁷⁴ Combination with 5-FC caused apoptosis, necrosis and reduced vascularisation and resulted in complete tumour regression as well as a reduction in metastases, indicating a distant bystander effect.

It seems likely that the combination of pPPE-CD with an additional prostate-specific UPRT expressing vector would increase the efficacy of this treatment strategy while still maintaining the many advantages of pPPE-CD in relation to radiation therapy. In fact, higher levels of 5-FdUMP as a result of UPRT activity would be likely to enhance the radiosensitizing effect of CD/5-FC due to increased blockage of DNA repair.

As discussed in section 1.9.1, GCV is a radiosensitizer as it prevents DNA replication, thereby preventing DNA repair following radiation damage.¹⁴⁴ Blackburn *et al.* tested a double suicide gene therapy system in the prostate tumour cell line PC3.²⁷⁵ This study looked at the effect of expression of the CD/TK fusion gene (under the control of the CMV promoter) when combined with prodrug and radiation treatment. GCV and/or 5-FC were introduced 24 hours before irradiation following transfection of the cells with the vector (at a multiplicity of infection of 10 pfu/cell). This resulted in a rise in radiosensitivity compared to radiation treatment alone. The most significant increase was observed when both prodrugs were administered. Similar effects on radiosensitivity have been observed in other tumour cell types when transfected with a CD/TK fusion gene, including colorectal carcinoma cells and adenoid cystic carcinoma cells.^{276, 277}

8.6 Potential of pPPE-CD/5-FC in the treatment of advanced and CRPC prostate cancer

The aim of this research was to test pPPE-CD/5-FC as a potential treatment for localised prostate cancer with a possible role as an adjuvant to radiation therapy. However, this GDEPT system may also have potential for the treatment of patients

with locally recurrent disease alongside androgen suppression therapy or for those patients with CRPC. It may seem counterintuitive to give pPPE-CD/5-FC treatment, which relies on functional androgen for its activation, alongside or after androgen suppression treatment but medical or surgical castration of those with recurrent disease has been shown to reduce DHT levels in the prostate by only 75%.²⁷⁸ The levels of DHT that remain (0.5 – 1 ng/g) have been shown to be sufficient to activate AR and in turn, AR-responsive genes.²⁴⁰

CRPC occurs at a median of 2-3 years after initiation of androgen deprivation therapy and the average survival is only 16-18 months.³⁸ It is characterised by a rise in PSA levels, despite continued treatment, and subsequent metastasis of the tumour.²⁷⁹ Formally, CRPC was known as androgen independent prostate cancer but this term is becoming less common as it is now recognised that the progression to this form of the disease is likely to involve a number of mechanisms, some of which are androgen dependent (discussed in section 1.3). In CRPC, AR signalling may be modulated by AR overexpression, mutations, changes in the levels of co-regulators or steroidogenic enzymes or de novo intraprostatic androgen synthesis.^{39, 237} The majority of CRPC tumours are AR-positive²⁸⁰ and the AR is structurally intact and capable of binding androgens.²⁸¹ In fact, AR is over-expressed in most cases of CRPC.

Measurable PSA expression and active AR in CRPC indicate that pPPE-CD/5-FC could have potential in this form of prostate cancer also. Previously, an adenovirus expressing thymidine kinase under the control of the PSA promoter was tested in LNCaP and C4-2, a castration-resistant form of LNCaP.²⁸² The vector had high expression levels in C4-2 in the absence of androgen and resulted in cell kill when combined with the prodrug acyclovir. *In vivo*, this GDEPT system inhibited C4-2 tumour growth and PSA expression in castrated mice.

It seems likely that cross-talk between AR and HIF-1 would still be active at the low DHT concentrations found in patients with CRPC, which are equivalent to about 0.1 nM DHT.²⁸³ Mitani *et al.* demonstrated AR transactivation in LNCaP at DHT concentrations of 0.05 and 0.1 nM, including the induction of PSA expression which was further increased by hypoxia.²¹² Hypoxia is a characteristic of CRPC tumours. Castration in rats has been shown to result in hypoxia in the surrounding

ventral prostate tissue due to decreased blood supply.^{284, 285} In LNCaP cells, oxidative stress upregulated the transcription factor Twist1, which in turn upregulates AR.²⁸⁶ This was suggested to contribute to the progression to castration resistance. In CRPC patient samples there is strong HIF-1 α expression.²⁸⁷

Bicalutamide is an example of an AR antagonist, prolonged treatment with which results in resistance. In a study by Kawata *et al.*, a bicalutamide-resistant LNCaP subline was created and was found to be hyper-sensitive to DHT, with proliferation stimulated by levels ten times lower than for LNCaP.³⁹ The cell line was capable of forming tumours in castrated mice, secreted PSA and showed AR overexpression. Bicalutamide has been shown to induce hypoxia-related gene expression in LNCaP which may play a role in progression to CRPC.²⁸⁸

Within the Prostate Molecular Oncology Research Group, Dr. Laure Marignol and Dr. Ruth Foley attempted to develop a bicalutamide resistant cell line by maintaining 22Rv1 cells in CD-FBS media with 100 nM DHT and 10 μ M bicalutamide (22RV1-BR) and age-matched control cells in media with 100 nM DHT only (22RV1-AMC). The protocol lasted for 40 weeks with weekly passaging. This cell line was a potential model in which to test the efficacy of pPPE-CD/5-FC treatment in a bicalutamide resistant environment. It was planned that CD expression levels would be measured in these two cell lines in response to high (1000nM) and much lower (0.1 – 1 nM) concentrations of DHT, followed by assays for cell kill when the vector was combined with 5-FC. In an attempt to elucidate the mechanism of bicalutamide resistance, AR expression levels would be measured through Western blotting and qRT-PCR and PSA expression levels would be compared for a range of DHT concentrations.

Before beginning these experiments clonogenic assays were carried out to confirm the bicalutamide resistant status of the cell line. Unfortunately these failed to show any difference in bicalutamide response between the proposed resistant 22Rv1 cells and the age-matched controls (Appendix H). This result meant the planned experiments using these cell lines could no longer take place in the time-frame of this research project. Future work could involve a second attempt to develop a bicalutamide resistant subline of 22Rv1. Also, it would be interesting to test pPPE-CD/5-FC in a castration resistant subline of LNCaP. If one of the suggested AR-

dependent mechanisms of progression to CRPC is playing a role, pPPE-CD should be activated in these cell lines at lower concentrations of DHT than are required for the parent LNCaP cell line.

8.7 Future development of GDEPT strategies

For successful transition of gene therapy to the clinic, ways to monitor transgene expression to assess the efficacy of the therapy in the patients as well as efficient and effective methods of gene delivery will be necessary. Several strategies have been used to achieve these goals and are discussed below.

8.7.1 Improved delivery (transductional targeting)

Adenoviruses remain the most widely used vector in gene therapy clinical trials. Entry of the virus occurs by endocytosis, when fibre proteins found on all 12 vertices of its icosahedral capsid interact with receptors on the cell surface.^{289, 290} Of the serotypes of adenovirus, the most commonly investigated is Ad type 5 (Ad5) of subgroup C.²⁹¹ Cell entry by this subgroup, through binding of the vector, has been shown to be initially dependent on cell surface expression of CAR.^{292, 293} This is accompanied by an interaction between the penton base of the fibre protein with the vitronectin receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, on the cell surface. Although the presence of the vitronectin receptors is not necessary, it greatly improves entry efficiency. Together, these interactions lead to viral entry.^{292, 293}

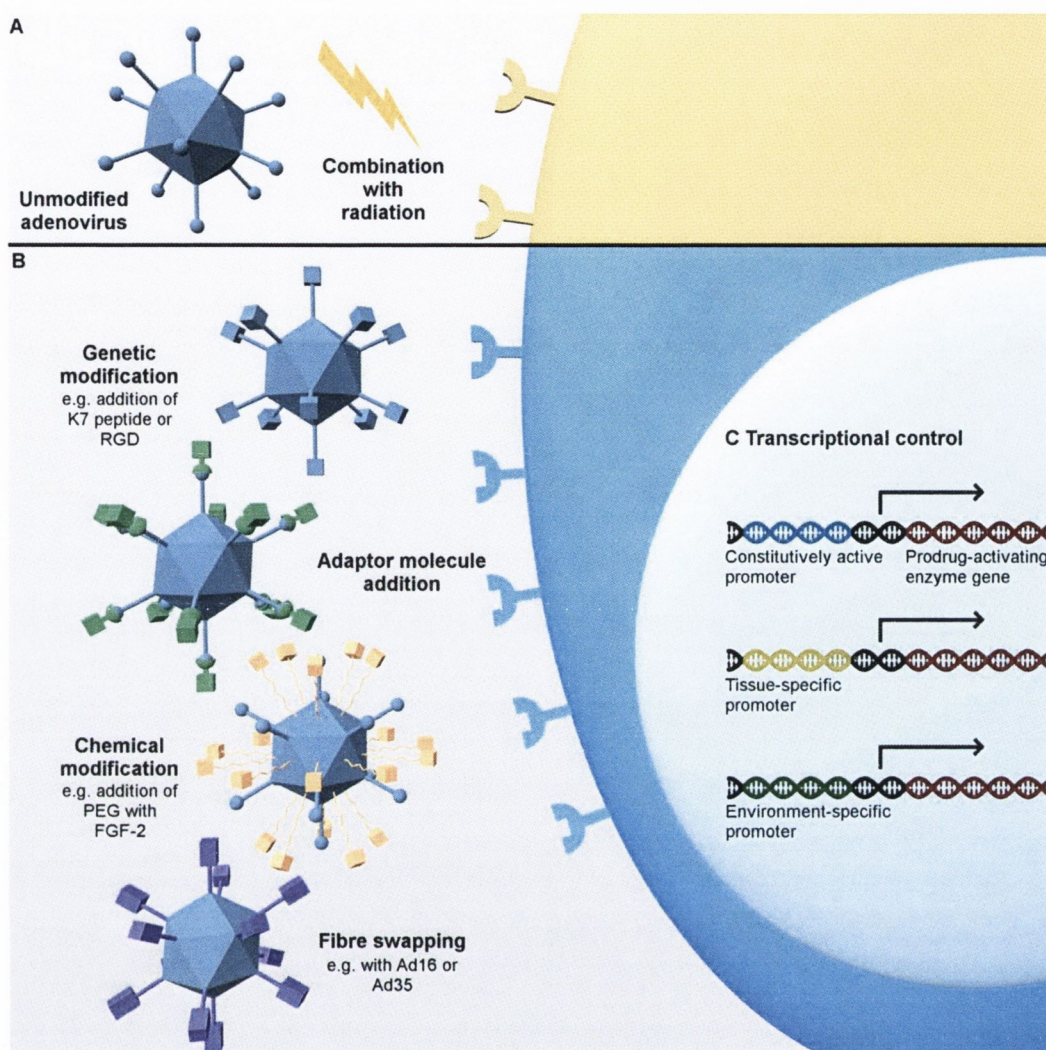


Figure 8.1: Transductional and transcriptional targeting of GDEPT. A) Radiation has been shown to increase the level of uptake of the adenovirus into mammalian cells and tumour xenografts. B) Manipulation of the adenovirus capsid allows it to bind to receptors other than CAR. This can be achieved through genetic modification, adaptor molecule addition, chemical modification or the replacement of the fibres with those from alternative CAR-independent members of the adenovirus family. C) The use of specific promoters allows for tissue/tumour targeting of expression of the prodrug activating enzyme and therefore targeted cell death. This may reduce the risk of toxic side effects.

An improvement in the transduction efficiency of adenoviral vectors is desirable. The increase in vector uptake when radiation therapy is combined with vector administration has been previously mentioned (section 1.9.2); however other ways to improve delivery are also being investigated. There are a number of issues that need to be addressed. Many cell types do not, in fact, express CAR at high levels, including skeletal and smooth muscle cells, dendritic cells and, haematopoietic stem cells.²⁹⁴ The low degree of expression means the necessary dose of vector is large, leading to a higher number of side effects. Although CAR may not be present on the cells of interest, its expression in general is widespread, as is that of the secondary and third receptors of Ad.²⁹⁴ This presents another problem – nonspecific entry of the vector causing toxicity. In the case of prostate cancer, Rauen *et al.* used a polyclonal antibody against human CAR to examine its levels in normal prostate tissue as well as primary and metastatic tumour samples.²⁹⁵ Examination of paraffin-embedded tissue showed that expression levels of CAR were high in the luminal and lateral cell membrane of normal prostate samples. However, the number of cells expressing CAR, as well as the strength of the expression, decreased with increasing Gleason grade for the prostate tumour samples. Metastatic samples were found to have high levels of CAR expression. Therefore the Gleason Grade and stage of cancer growth may be an important factor in determining the degree of successful viral delivery.

A number of approaches to overcome these problems have been investigated, through the manipulation of the adenovirus to create a more efficient, targeted delivery system (discussed in detail by Campos *et al.*²⁹⁶). One approach is to genetically alter the capsid of the virus to direct it to particular cell types and allow attachment of the fibre proteins to other receptors on the cell surface (Figure 8.1). In this strategy, specific peptides are inserted into the fibre knob, for example, poly-lysine residues (K7 peptide) can be inserted into the C-terminus and an Arg-Gly-Asp (RGD) peptide inserted into the HI loop.^{297, 298} This increases the tropism of these vectors and allows them to enter cells not normally susceptible to transduction. Other approaches have involved modification of different parts of the viral capsid, including the hypervariable region 5 (HVR5) loop of the hexon, the C-terminus of protein IX and the penton base.²⁹⁹⁻³⁰¹

Another way to manipulate vector targeting involves the use of bispecific or bifunctional adaptor molecules, a capsid-binding domain joined to a cell-binding ligand such as fibroblast growth factor-2 or epidermal growth factor.^{302, 303} However, this technique is limited by batch-to-batch variation in the preparation of the fusion proteins and the weak non-covalent protein-protein binding of the molecules to the capsid, which may be unable to compete *in vivo*.²⁹⁶

Chemical modification of the capsid is another option for adapting adenovirus entry. One chemical used is polyethylene glycol (PEG) which has the advantage of lengthening the time the vector is present in the blood, stopping neutralization by antibodies as well as lessening the innate immune response to the vectors.³⁰⁴⁻³⁰⁶ However this modification of the vector also results in decreased infectivity. This can be counteracted through the attachment of peptides like FGF-2 to PEG, improving transgene expression and targeting.³⁰⁷ Alternatives to PEGylation include addition of poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA) to the viral surface.³⁰⁸

Attempts to circumvent the requirement of adenovirus type 5 (Ad5) for CAR to bind to the cell surface have taken advantage of the variety in the adenoviridae family.²⁹⁶ Tropism of the vector can be altered through the swapping of fibres of the Ad5 with those of members of subgroup B to create fibre-chimeric vectors. Examples include Ad16 and Ad35.^{291, 294, 309, 310} Unlike subgroups A, C, D, E and F, most of whose members need CAR, subgroup B uses other receptors: CD46, CD80/CD86 and “receptor X”.³¹¹⁻³¹⁶ Therefore, the vectors become CAR independent, allowing high levels of transduction of a wide range of cell types.

8.7.2 *Non-invasive imaging of transgene activity*

The ability to image transgene activity is crucial to the progress of GDEPT. Research is being carried out to develop non-invasive methods of monitoring transgene expression to help assess the success of the treatment. Liu *et al.* describe their continuing work on examining transgene expression with the reporter gene *lacZ*.¹⁷⁹ This gene encodes β -galactosidase (β -gal), the activity of which is detected using a substrate of the enzyme, 2-fluoro-4-nitrophenyl β -D-galactopyranoside (OFPNPG). Using ¹⁹F nuclear magnetic resonance, the cleavage of OFPNPG can be detected. This was tested in *lacZ*-expressing PC3 prostate tumour xenografts in

mice. This work describes a novel method for confirming transgene expression with possible application in GDEPT. Continued investigation is being carried out to develop reporters with lower toxicity.

A number of studies have examined the potential for imaging of transgene activity using a single gene as the reporter as well as the therapeutic gene. One of the most recent examples of this was research published by Gade *et al.* which used magnetic resonance spectroscopy imaging (MRSI) to examine the *in vivo* expression of the fusion gene encoding CD-UPRT in CD-UPRT⁺ and CD-UPRT⁻ W256 xenografts in nude mice.³¹⁷ In mixed tumours (consisting of both wild type and CD-UPRT⁺ W256 cells) this technique was able to detect heterogeneity in enzyme activity.

A second reporter gene system involves HSVtk and uses the therapeutic gene itself. HSVtk phosphorylates certain labelled probes, such as the acycloguanosines (e.g. ganciclovir, penciclovir), preventing movement of the compounds back across the cell membrane, thereby trapping them and allowing their visualisation using positron emission tomography (PET).³¹⁸⁻³²¹ Therefore, using a suitable substrate, the localisation and magnitude of HSVtk gene expression can be assessed. To enhance efficacy of the enzyme as a reporter, the mutant gene HSV1-sr39tk was developed, the product of which can more efficiently act on fluorinated acycloguanosine substrates, increasing the sensitivity of the PET reporter system.³¹⁹ Importantly, an improvement in enzyme activity on acycloguanosines means an increase in cytotoxicity and more effective GDEPT.^{319, 320}

An alternative reporter gene system that uses nuclear medicine imaging involves a mutated version of the dopamine type 2 receptor (D2R) gene, which can bind to its ligand but is incapable of activating downstream signalling. Using this system and the radiolabelled probe ¹⁸F-fluoroethylspiperone (FESP), *in vivo* and *in vitro* assays have shown that measurement of FESP build up using PET is a true indicator of gene expression in the tissues of interest.^{322, 323} In work by Kummer *et al.*, tests were carried out using a universal HSV amplicon vector that expressed both HSVtk and D2R in a bicistronic cassette in a human glioma model. The PET studies demonstrated that the D2R system gave as much information on gene expression *in vivo* as the HSVtk gene system.³²⁴

The reporter gene systems (HSVtk and D2R) that use PET can be expensive and complex.³²⁰ Therefore, some systems have been developed that require a γ -camera instead of a costly PET unit; examples are the somatostatin reporter (SSTr) 2 gene and ¹¹¹Inoctreotide, norepinephrine transporter gene and ¹³¹I-metaiodobenzylguanidine, and the human sodium iodide symporter (hNIS) gene and radioiodines or ^{99m}Tc-pertichnetate. Of these, it has been suggested that the NIS gene system is the most straightforward.³²⁰

The sodium iodide symporter (NIS) is a transmembrane glycoprotein consisting of 13 transmembrane segments, an extracellular N-terminus domain and an intracellular C-terminus.³²⁵ It is found naturally in a number of human tissues: mainly the thyroid but in others too, including the stomach, salivary glands, mammary glands and extraocular muscles.³²⁶ It is a member of the sodium/solute symporter family but can transport many anions in addition to iodide.³²⁷ Its ability to transport radioactive iodide and other radiolabelled anions such as ^{99m}TcO₄⁻ and ⁷⁶Br⁻ means it has potential for use in diagnostics and as a reporter gene for GDEPT.^{327, 328} As it is a human gene it should not cause an adverse immune response. Its substrates are easily available, as is information on their metabolisms. Unlike D2R and HSVtk, labelling of the substrates of NIS is stable. However the use of the NIS gene system is limited in specific tissues by its endogenous expression which would reduce sensitivity and specificity (although this should not affect its potential use in the prostate). It is also restricted by the swift loss of iodide from hNIS-expressing cells..^{320, 329}

Recently a phase I study has examined the feasibility of using a therapeutic adenovirus, monitored noninvasively through the utilization of the sodium iodide transporter.³³⁰ Replication-competent Ad5-yCD/*mutTK*_{SR39}*rep*-hNIS was injected into the prostate of men with clinically localised prostate cancer. This was followed by administration of the radioactive tracer sodium pertechnetate (Na^{99m}TcO₄), allowing imaging of NIS gene expression using single photon emission-computed tomography (SPECT). Using this method, Barton *et al.* were able to calculate the volume, total amount and persistence of gene expression in the prostate. This gene expression was not detected in any tissue outside the prostate and none of the

adverse events (98% Grade 1 or 2) were deemed to be caused by the nuclear imaging.

Barton *et al.* also highlighted the advantages of this particular reporter gene system. Since the hNIS gene is under the control of cytomegalovirus promoter, which is ubiquitous, NIS expression is not restricted to any tissue in particular. This is in contrast to reporter genes that are under the control of prostate specific promoters and therefore could not give information about the spread of the virus outside the prostate. In the case of this particular adenovirus, Barton *et al.* noted that a variety of imaging techniques could be utilized as CD and HSVtk can also be imaged noninvasively as previously discussed. Therefore, the most suitable reporter system for the tissue in question could be chosen.

8.8 Conclusion

Following on from previous research demonstrating the androgen-inducibility of pPPE-CD, it was hypothesised that hypoxia would further increase its activity. This was successfully demonstrated, as was the radiation-responsive nature of CD expression. Therefore, this GDEPT system has the advantage of being androgen, hypoxia and radiation-inducible, allowing for localised, targeted chemotherapy in the prostate. The highest levels of CD expression and consequently 5-FU should be in those cells likely to be resistant to current radiation therapy. This should result in toxicity of the chemotherapeutic drug, as well as increased radiosensitization which may help reduce the number of surviving tumour cells. This project puts forward evidence for the potential of a combined treatment strategy involving pPPE-CD/5-FC and radiation treatment of the tumour.

The development of *in vitro* models in which to test pPPE-CD activity was also successful. Although 22Rv1-ARhi had much lower levels of PSA protein and mRNA than the parent cell line 22Rv1, when transfected with pPPE-CD, CD expression was strong. This demonstrates that despite the fact that the observed PSA expression levels in 22Rv1-ARhi do not follow expected trends, a biologically relevant environment that is responsive to the pPPE-CD/5-FC system has been created. Formation of 22Rv1 spheroids using a novel technique resulted in a model of prostate cancer which shows great promise for improvement over traditional monolayer systems. pPPE-CD/5-FC efficacy was also demonstrated in this context

and further characterisation of the 22Rv1 spheroid response to the pPPE-CD/5-FC system would be both informative and potentially clinically useful. Further development of this *in vitro* spheroid model, as well as development of spheroid models in other cancer cell lines using the HC media system, represents an exciting avenue for future research.

Although the current treatments for localised prostate cancer are relatively effective, some patients will suffer recurrence and progress to metastatic prostate cancer, for which there are no curative options. Any improvement in the current therapeutic strategies for both localised and recurrent tumours would be beneficial. Research into GDEPT has demonstrated it is a potential avenue for development of improved treatment, with successful Phase I/II trials involving a host of cancer types. It is hoped that the research described within this thesis will positively contribute to the growing body of knowledge concerning GDEPT and associated research methods, and that such GDEPT research will be fruitful in bringing about improved patient outcome following treatment of prostate cancer.

Appendices

Appendix A In silico analysis of endogenous control genes for qRT-PCR

Table A.1: HIF-1 α and HIF-1 β TFBS in proximal promoters of endogenous controls

Gene	HIF-1 α TFBS	PSSM Threshold	HIF-1 β TFBS	PSSM Threshold
<i>18S rRNA</i>	N/A	-	N/A	-
<i>ACTB</i>	P	0.75	P	0.7
<i>B2M</i>	A	-	A	-
<i>GAPDH</i>	P	0.85	P	0.85
<i>GUSB</i>	A	-	A	-
<i>HMBS</i>	P	0.85	A	-
<i>HPRT1</i>	A	-	P	0.7
<i>IPO8</i>	N/A	-	N/A	-
<i>PGK1</i>	P	0.85	P	0.75
<i>POLR2A</i>	P	0.8	A	-
<i>PPIA</i>	A	-	A	-
<i>RPLP0</i>	P	0.7	A	-
<i>TBP</i>	P	0.8	A	-
<i>TFRC</i>	P	0.7	P	0.75
<i>UBC</i>	P	0.7	A	-
<i>YWHAZ</i>	P	0.75	A	-

TFBS, transcription factor binding site; P/A, Present/Absent; N/A, not present in the database; PSSM threshold, most stringent threshold at which the HRE was detected.

Appendix B Plasmid map



Figure B.1: pCMV-CD vector showing location of CMV promoter and enhancer and CD open reading frame. Numbers represent the positions in the GenBank sequence.

Appendix C Insert sequences

PSA promoter and enhancer sequence

The yellow sequence is the PSA promoter and the red is the PSA enhancer. ARE III is highlighted in pink and the putative HRE is highlighted in green.

1	GGTAGCCTTG	CAGAAGTTGG	TCGTGAGGCA	CTGGGCAGGT	AAGTATCAAG
51	GTTACAAGAC	AGGTTTAAGG	AGACCAATAG	AAACTGGGCT	TGTCGAGACA
101	GAGAAGACTC	TTGCGTTTCT	GATAGGCACC	TATTGGTCTT	ACTGACATCC
151	ACTTTGCCTT	TCTCTCCACA	GGTGTCCACT	CCCAGTTCAA	TTACAGCTCT
201	TAAGGCTAGA	GTACTTAATA	CGACTCACTA	TAGGCTAGCC	TCGAGAATTC
251	ACGCGTGGTA	CCTCTAGAGT	CGACCCGGGC	GGCCGCCAGT	GTGATGGATA
301	TCTGCAGAAAT	TCGCCCTTCG	GAATGAGCTC	GCACAGATAG	AGTGTACAGG
351	TCTGGAGAAC	AAGGAGTGGG	GGGTTATTGG	AATTCCACAT	TGTTTGCTGC
401	ACGTTGGATT	TTGAAATGCT	AGGGAACTTT	GGGAGACTCA	TATTTCTGGG
451	CTAGAGGATC	TGTGGACCAC	AAGATCTAAT	CTAGCTGATA	TAGTGTGGCT
501	CAAAACCTTC	AGCACAAATC	ACACCGTTAG	ACTATCTGGT	GTGGCCCAAA
551	CCTTCAGGTG	AACAAAGGGA	CTCTAATCTG	GCAGGATATT	CCAAAGCATT
601	AGAGATGACC	TCTTGCAAAG	AAAAAGAAAT	GGAAAAGAAA	AAGAAAGAAA
651	GGAAAAA	AAAAAAAAG	AGATGACCTC	TCAGGCTCTG	AGGGGAAACG
701	CCTGAGGTCT	TTGAGCAAGG	TCAGTCCTCT	GTTGCACAGT	CTCCCTCACA

751	GGGTCATTGT	GACGATCAAA	TGTGGTCACG	TGTATGAGGC	ACCAGCACAT
801	GCCTGGCTCT	GGGGAGTGCC	GTGTAAGTGT	ATGCTTGAC	TGCTGAATGC
851	TTGGGATGTG	TCAGGGATTA	TCTTCAGCAC	TTACAGATGC	TCATCTCATC
901	CTCACAGCAT	CACTATGGGA	TGGGTATTAC	TGGCCTCATT	TGATGGAGAA
951	AGTGGCTGTG	GCTCAGAAAG	GGGGGACCAC	TAGACCAGGG	ACACTCTGGA
1001	TGCTGGGGAC	TCCAGAGACC	ATGACCACTC	ACCAACTGCA	GAGAAATTAA
1051	TTGTGGCCTG	ATGTCCCTGT	CCTGGAGAGG	GTGGAGGTGG	ACCTTCACTA
1101	ACCTCCTACC	TTGACCCTCT	CTTTTAGGGC	TCTTTCTGAC	CTCCACCATG
1151	GTACTIONGAC	CCCATTGTAT	TCTGTACCCT	CTTGACTCTA	TGACCCCCAC
1201	TGCCCCACTGC	ATCCAGCTGG	GTCCCCCTCT	ATCTCTATTC	CCAGCTGGCC
1251	AGTGCAGTCT	CAGTGCCAC	CTGTTTGTCA	GTAACCTCTGA	AGGGGCTGAC
1301	ATTTTACTGA	CTTGCAAACA	AATAAGCTAA	CTTTCCAGAG	TTTTGTGAAT
1351	GCTGGCAGAG	TCCATGAGAC	TCCTGAGTCA	GAGGCAAAGG	CTTTTACTGC
1401	TCACAGCTTA	GCAGACAGCA	TGAGGTTCAT	GTTACATTA	GTACACCTTG
1451	CCCCCCCCAA	ATCTTGTAGG	GTGACCAGAG	CAGTCTAGGT	GGATGCTGTG
1501	CAGAAGGGGT	TTGTGCCACT	GGTGAGAAAC	CTGAGATTAG	GAATCCTCAA
1551	TCTTATACTG	GGACAACTTG	CAAACCTGCT	CAGCCTTTGT	CTCTGATGAA
1601	GATATTATCT	TCATGATCTT	GGATTGAAAA	CAGACCTACT	CTGGA
1651		ATTGTCCTT	GACAGTAAAC	AAATCTGTTG	TAAGAGACAT
1701	TATCTTTATT	ATCTAGGACA	GTAAGCAAGC	CTGGATCTGA	GAGAGATATC
1751	ATCTTGCAAG	GATGCCTGCT	TTACAAACAT	CCTTGAAACA	ACAATCCAGA
1801	AAAAAAAAGG	TGTTGCTGTC	TTTGCTCAGA	AGACACACAG	ATAGCTGACA
1851	GAACCATGGA	GAATTGCCTC	CCAACGCTGT	TCAGCCAGAG	CCTTCCACCC
1901	TTGTCTGCAG	GACAGTCTCA	ACGTTCACC	ATTAAATACT	TCTTCTATCA
1951	CATCCTGCTT	CTTTATGCCT	AACCAAGGTT	CTAGGTCCC	ATCGACTGTG
2001	TCTGGCAGCA	CTCCACTGCC	AAACCCAGAA	TAAGGCAGCG	CTCAGGATCC
2051	AGATCTTTTT	ATGATGACAG	TAGCAATGTA	TCTGTGGAGC	TGGATTCTGG
2101	GTTGGGAGTG	CAAGGAAAAG	AATGTACTAA	ATGCCAAGAC	ATCTATTTCA
2151	GGAGCATGAG	GAATAAAAGT	TCTAGTTTCT	GGTCTCAGAG	TGGTGCAGGG
2201	ATCAGGGAGT	CTCACAATCT	CCTGAGTGCT	GGTGTCTTAG	GGCACACTGG
2251	GTCTTGAGC	GCAAAGGATC	TAGGCACGTG	AGGCTTTGTA	TGAAGAATCG
2301	GGGATCGTAC	CCACCCCCTG	TTTCTGTTTC	ATCCTGGGCG	TGTCTCCTCT
2351	GCCTTTGTCC	CCTAGATGAA	GTCTCCATGA	GCTACAGGGC	CTGGTGCATC
2401	CAGGGTGATC	TAGTAATTGC	AGAACAGCAA	GTACTIONGCTC	TCCCTCCCCT
2451	TCCACAGCTC	TGGGTGTGGG	AGGGGGTTGT	CCAGCCTCCA	GCAGCATGGG
2501	GAGGGCCTTG	GTCAGCCTCT	GGGTGCCAGC	AGGGCAGGGG	CGGAGTCCTG
2551	GGGAATGAAG	GTTTTATAGG	GCTCCTGGGG	GAGGCTCCCC	AGCCCCAAGC

Cytosine deaminase sequence

Positions 17-1519 correspond to positions 131-1633 of the GenBank sequence (accession number S56903).

1	GAATTCAGGC	TAGCAATGTC	GAATAACGCT	TTACAAACAA	TTATTAACGC
51	CCGGTTACCA	GGCGAAGAGG	GGCTGTGGCA	GATTCATCTG	CAGGACGGAA
101	AAATCAGCGC	CATTGATGCG	CAATCCGGCG	TGATGCCCAT	AACTGAAAAC
151	AGCCTGGATG	CCGAACAAGG	TTTAGTTATA	CCGCCGTTTG	TGGAGCCACA
201	TATTCACCTG	GACACCACGC	AAACCGCCGG	ACAACCGAAC	TGGAATCAGT
251	CCGGCACGCT	GTTTGAAGGC	ATTGAAGGCT	GGGCCGAGCG	CAAAGCGTTA
301	TTAACCCATG	ACGATGTGAA	ACAACGCGCA	TGGCAAACGC	TGAAATGGCA

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351      GATTGCCAAC GGCATTGAGC ATGTGCGTAC CCATGTCGAT GTTTCGGATG
401      CAACGCTAAC TGCGCTGAAA GCAATGCTGG AAGTGAAGCA GGAAGTCGCG
451      CCGTGGAATTG ATCTGCAAAT CGTCGCCTTC CCTCAGGAAG GGATTTTGTC
501      GTATCCCAAC GGTGAAGCGT TGCTGGAAGA GGC GTTACGC TTAGGGGCGAG
551      ATGTAGTGGG GCGGATTCCG CATTTTGAAT TTACCCGTGA ATACGGCGTG
601      GAGTCGCTGC ATAAAACCTT CGCCCTGGCG CAAAATACG ACCGTCTCAT
651      CGACGTTTAC TGTGATGAGA TCGATGACGA GCAGTCGCGC TTTGTCGAAA
701      CCGTTGCTGC CCTGGCGCAC CATGAAGGCA TGGGCGCGCG AGTCACCGCC
751      AGCCACACCA CGGCAATGCA CTCCTATAAC GGGGCGTATA CCTCACGCCT
801      GTTCCGCTTG CTGAAAATGT CCGGTATTAA CTTTGTGCGC AACCCGCTGG
851      TCAATATTCA TCTGCAAGGA CGTTTCGATA CGTATCCAAA ACGTCGCGGC
901      ATCACGCGCG TTAAAGAGAT GCTGGAGTCC GGCATTAACG TCTGCTTTGG
951      TCACGATGAT GTCTTCGATC CGTGGTATCC GCTGGGAACG GCGAATATGC
1001     TGCAAGTGCT GCATATGGGG CTGCATGTTT GCCAGTTGAT GGGCTACGGG
1051     CAGATTAACG ATGGCCTGAA TTTAATCACC CACCACAGCG CAAGGACGTT
1101     GAATTTGCAG GATTACGGCA TTGCCGCCGG AACAGCGCC AACCTGATTA
1151     TCCTGCCGGC TGAAAATGGG TTTGATGCGC TGCGCCGTCA GGTTCGGTA
1201     CGTTATTCGG TACGTGGCGG CAAGGTGATT GCCAGCACAC AACCGGCACA
1251     AACCACCGTA TATCTGGAGC AGCCAGAAGC CATCGATTAC AACGTTGAA
1301     CGACTGGGTT ACAGCGAGCT TAGTTTATGC CGGATGCGCG GTGAACGCCT
1351     TATCCGGCCT ACGTAGAGCA CTGAACTCGT AGGCCTGATA AGCGTAGCGC
1401     ATCAGGCAAT TCCAGCCGCT GATCTGTGTC AGCGGCTACC GTGATTCATT
1451     CCCGCCAACA ACCGCGCATT CCTCCAACGC CATGTGCAAA AATGCCTTCG
1501     CAGCGGCTGT CTGCCAGCTA GAGGATCC

```

Appendix D Cell lines standard curves for MTT analysis

The cell lines were seeded at various concentrations and an MTT assay was carried out once the cells had adhered to the plate. This allowed absorbance to be plotted against cell number and the slope of this line was used in subsequent experiments to normalise MTT results to the number of cells.

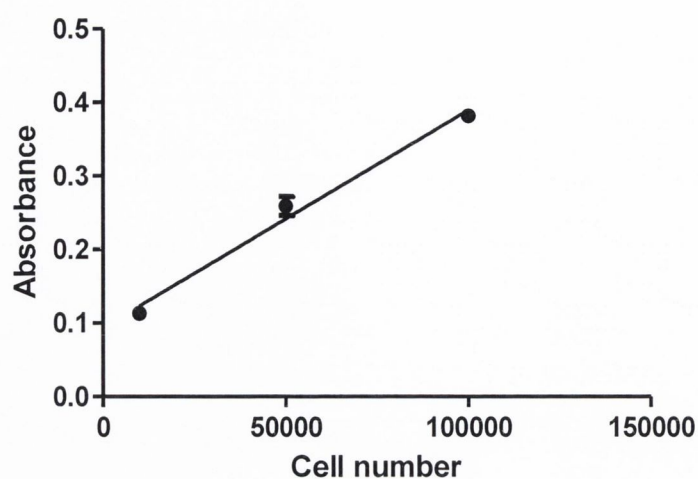


Figure D.1: 22Rv1 absorbance at a range of cell concentrations. Equation of the line, $y = 327981x - 29128$.

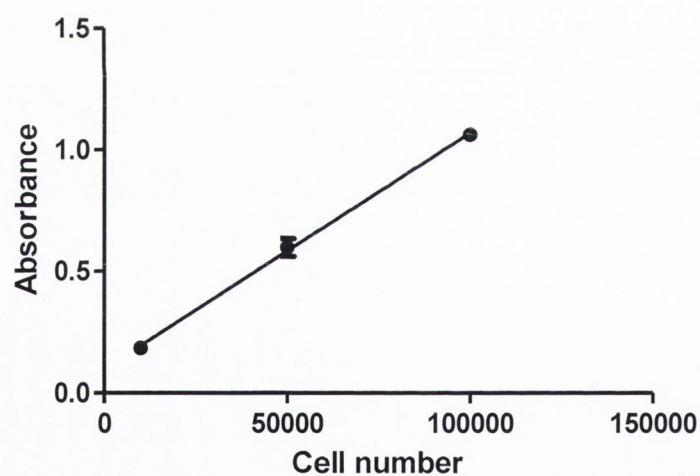


Figure D.2: LNCaP absorbance at a range of cell concentrations. Equation of the line, $y = 101313x - 9035.9$.

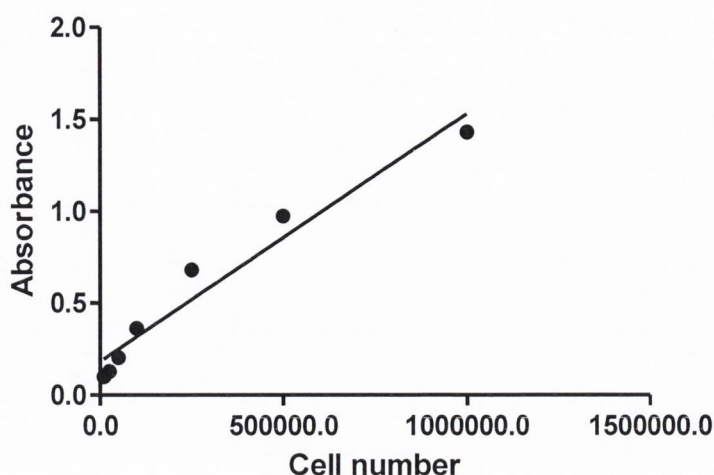


Figure D.3: 22Rv1-ARhi absorbance at a range of cell concentrations. Equation of the line, $y = 531909x - 61609$.

Appendix E Optimisation of transfection using TransFast

To optimise DNA and TransFast concentrations, a GFP expressing vector was used. The DNA and TransFast were combined as follows

- 1) 1µg DNA 3µl TransFast
- 2) 2.5µg DNA 7.5µl TransFast
- 3) 1µg DNA 6µl TransFast
- 4) 2.5µg DNA 15µl TransFast
- 5) 1µg DNA 9µl TransFast

Transfection was carried out as described in section 2.10. The following day the cells were observed using a fluorescent microscope and the levels of GFP expression and toxicity were compared. The optimal levels were observed for treatment 2, followed by treatment 1. 2.5µg DNA and 7.5µl TransFast were used for future transfection experiments.

Appendix F qRT-PCR

Before qRT-PCR analysis, the endogenous control gene was tested to ensure its suitability under the relevant treatment conditions. In addition, a ten-fold serial

dilution of cDNA (100 – 0.01 ng) was used to compare the efficiencies of the target gene and the endogenous control gene. The slope of the log of the input amount of cDNA plotted against the ΔC_T value for the two genes must be between -0.1 and 0.1 (i.e. the two probes must be of similar efficiency) for the $\Delta\Delta C_T$ method of quantifying relative gene expression to be accurate. The results for various genes used during the course of this project are shown in Figure F.1 - Figure F.3, along with the slope of the line.

For initial experiments under aerobic conditions, PGK1 was used. The C_T values for PGK1 were similar between the different cell lines used and were not affected by the treatment conditions (Table F.1).

Table F.1: Average CT for PGK1 for different cells lines under various treatment conditions

Cell line	Growth conditions	Average C_T ± SD
LNCaP	Complete media	19.83±0.18
LNCaP	Charcoal stripped media	20.58±0.26
LNCaP	Charcoal stripped media & 1000nM DHT	20.37±0.29
22Rv1	Complete media	20.55±0.05
22Rv1	Charcoal stripped media	20.72±0.27
22Rv1	Charcoal stripped media & 1000nM DHT	20.58±0.24
22Rv1-ARhi	Complete media	21.4±0.34
22Rv1-ARhi	Charcoal stripped media	21.72±0.19
22Rv1-ARhi	Charcoal stripped media & 1000nM DHT	21.03±0.24

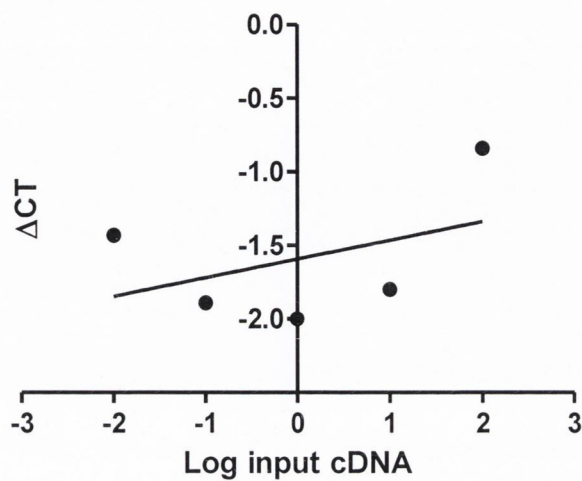


Figure F.1: The log of the input amount of cDNA versus ΔCT for AR and PGK1.
Slope = 0.1270 ± 00.1559

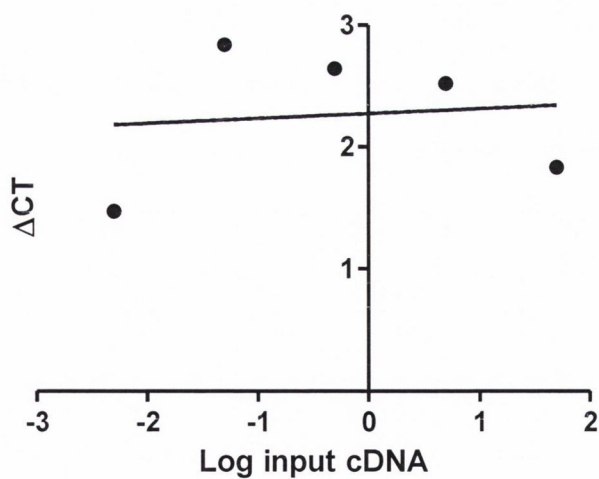


Figure F.2: The log of the input amount of cDNA versus ΔCT for AR and PGK1.
Slope = 0.1270 ± 00.1559

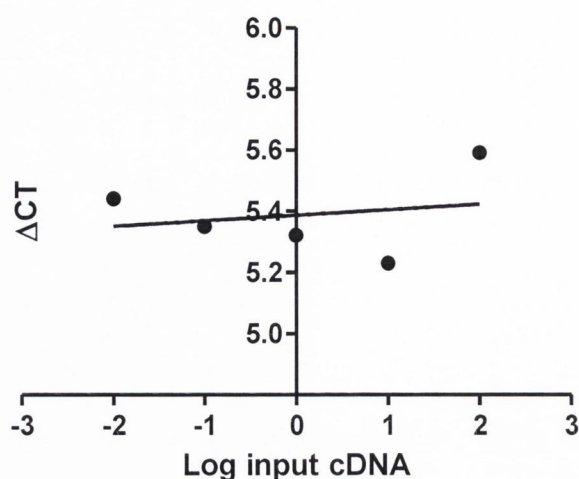


Figure F.3: The log of the input amount of cDNA versus ΔCT for FKBP51 and ACTB. Slope = 0.04 ± 0.2115

Appendix G Clonogenic assays to test 5-FC activation in hypoxia

22Rv1 and DU145 were treated with 0, 1 or 5 mM 5-FC for 48 and 72 hours to compare the toxicity of the prodrug under aerobic (21% oxygen) and hypoxic (0.5%) conditions. For 22Rv1 cells, 1 mM 5-FC treatment resulted in a significant decrease in colony numbers under hypoxic compared to aerobic conditions after 48 hours ($p < 0.05$, Figure G.1) and 72 hours of treatment ($p < 0.01$, Figure G.2). This was not the case for DU145 cells where there was no significant difference (Figure G.3 and Figure G.4).

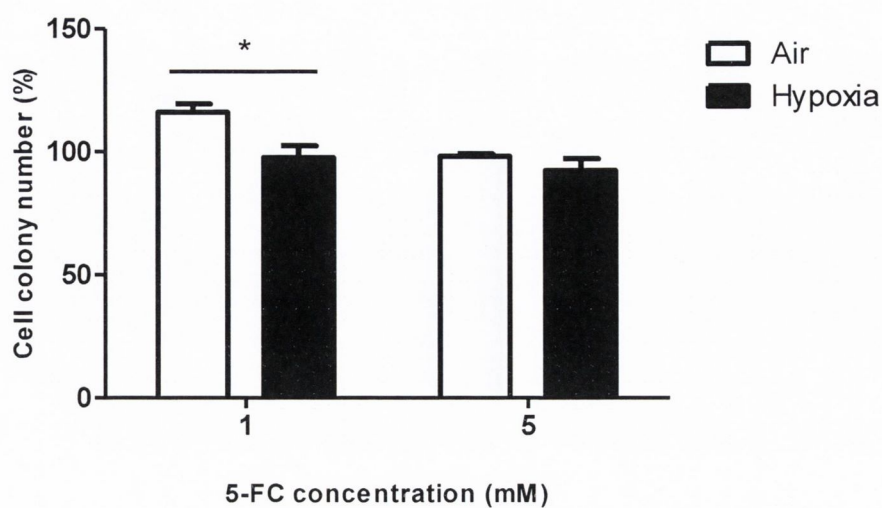


Figure G.1: 22Rv1 cell colony number following a 48 hour treatment of 5-FC expressed as a percentage of the corresponding untreated controls. Values represent the mean of three replicates $\pm$ standard error of the mean. *, $p < 0.05$.

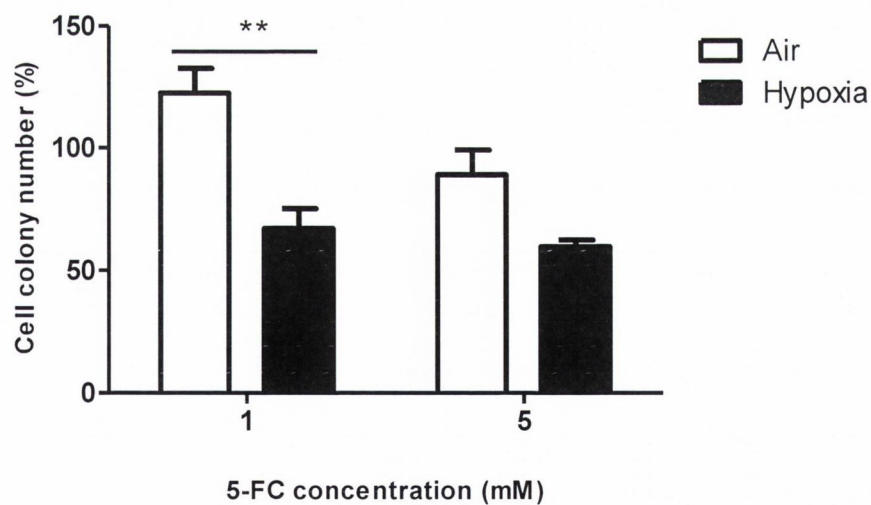


Figure G.2: 22Rv1 cell count following a 72 hour treatment of 5-FC expressed as a percentage of the corresponding untreated controls. Values represent the mean of three replicates $\pm$ standard error of the mean. **, $p < 0.01$.

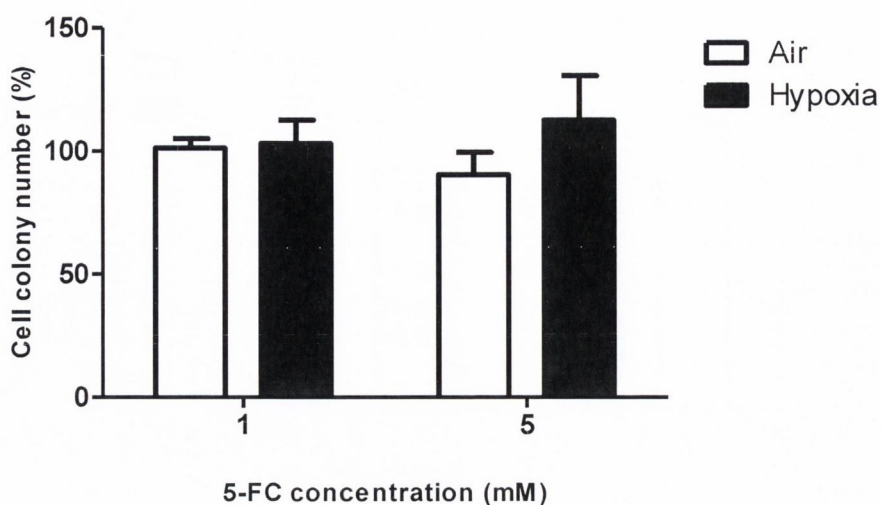


Figure G.3: DU145 cell count following a 48 hour treatment of 5-FC expressed as a percentage of the corresponding untreated controls. Values represent the mean of three replicates $\pm$ standard error of the mean.

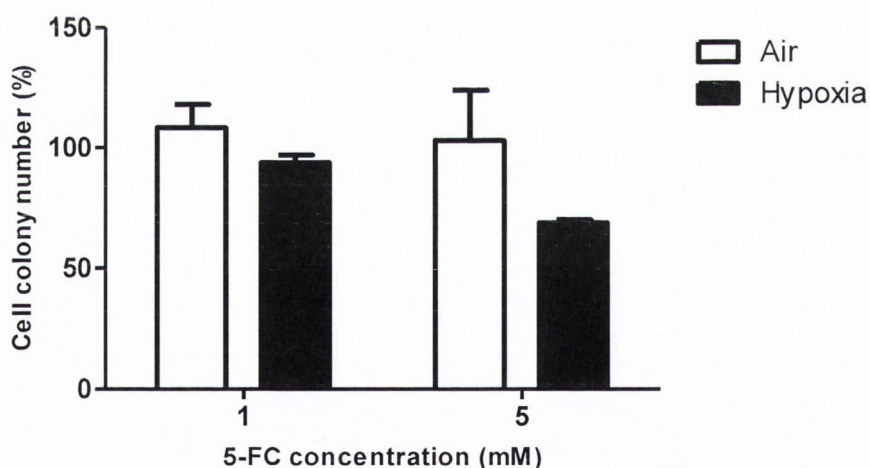


Figure G.4: DU145 cell count following a 72 hour treatment of 5-FC expressed as a percentage of the corresponding untreated controls. Values represent the mean of three replicates $\pm$ standard error of the mean.

Appendix H Bicalutamide

In the Prostate Molecular Oncology Group, attempts were made to produce a bicalutamide resistant subline of 22Rv1 (22Rv1-BR). However, upon testing using clonogenic assay, no difference was observed in the response to bicalutamide between 22Rv1-BR and the age-matched control cell line (Figure H.1).

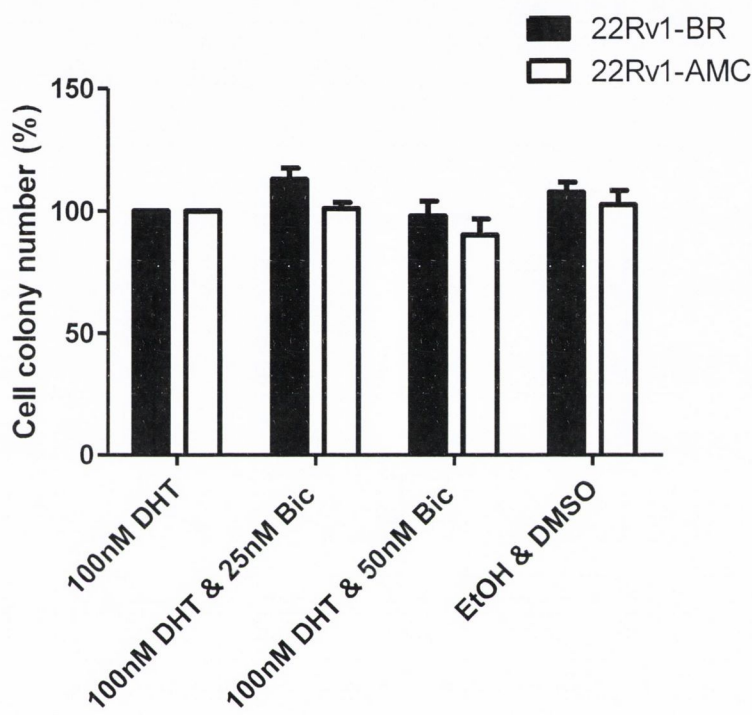


Figure H.1: Colony number following a four day treatment with bicalutamide, expressed as a percentage of the corresponding controls treated with DHT alone. Values represent the mean of three replicates $\pm$ standard error of the mean. 22Rv1-BR, bicalutamide resistant cell line; 22Rv1-AMC, age-matched control; Bic, bicalutamide; EtOH, ethanol.

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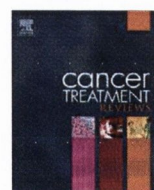
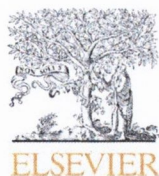
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New Drugs

Clinical potential of gene-directed enzyme prodrug therapy to improve radiation therapy in prostate cancer patients

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ARTICLE INFO

Article history:

Received 24 November 2010

Received in revised form 8 March 2011

Accepted 16 March 2011

Keywords:

Gene-directed enzyme prodrug therapy

GDEPT

Radiation therapy

Prostate cancer

ABSTRACT

Despite the advances in prostate cancer diagnosis and treatment, current therapies are not curative in a significant proportion of patients. Gene-directed enzyme prodrug therapy (GDEPT), when combined with radiation therapy, could improve the outcome of treatment for prostate cancer, the second leading cause of cancer death in the western world. GDEPT involves the introduction of a therapeutic transgene, which can be targeted to the tumour cells. A prodrug is administered systemically and is converted to its toxic form only in those cells containing the transgene, resulting in cell kill. This review will discuss the clinical trials which have investigated the potential of GDEPT at various stages of prostate cancer progression. The advantages of using GDEPT in combination with radiotherapy will be examined, as well as some of the recent advances which enhance the potential utility of GDEPT.

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Introduction

Current clinical practice utilises two radical forms of therapy for organ-confined prostate cancer with curative intent. These are surgery in the form of retropubic radical prostatectomy (RRP) and radiation therapy, either radical external beam radiotherapy (EBRT) or brachytherapy. The efficacy of treatment is quite variable and is strongly related to patient risk stratification. In the case of EBRT, patients with low-risk prostate cancer (Stage $\leq$ T2a, Gleason score $\leq$ 6, PSA $<$ 10 ng/ml) have long-term disease-free survival rates above 80%.¹ However, patients with intermediate (stage T2b or Gleason score = 7 or PSA 10–20 ng/ml) or high-risk (Stage T2c or Gleason score $\geq$ 8 or PSA $>$ 20 ng/ml) forms of the disease have much lower survival rates (25–75%).² The scope for further improvement of these survival rates by traditional radiation dose

escalation strategies or longer duration concomitant androgen blockade is likely to be modest.

Gene-directed enzyme prodrug therapy (GDEPT), also known as suicide gene therapy, enzyme-activating prodrug therapy and gene prodrug activation therapy, has therefore been proposed as a possible way of improving the outcome of radiotherapy for prostate cancer (see Fig. 1).³ Multiple enzyme/prodrug systems are currently being tested for their potential to augment prostate cancer treatment. This review will discuss the potential of using the cytosine deaminase/5-fluorocytosine (CD/5-FC) and herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) GDEPT systems alongside radiotherapy for the management of prostate cancer patients.

Gene-directed enzyme prodrug therapy

GDEPT involves the administration of an enzyme-encoding transgene. A relatively low or non-toxic prodrug is introduced into the tissue, in this case the prostate, and is converted to its toxic form by the enzyme, leading to cell kill.⁴

Cytosine deaminase/5-fluorocytosine prodrug system

5-Fluorouracil (5-FU) is currently used as a chemotherapeutic drug, in particular for the treatment of colorectal cancer. However, to be effective, high doses of the drug are required resulting in undesirable side-effects.⁵ Therefore, a possible way to improve

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on this therapy is to introduce the drug using GDEPT. 5-Fluorocytosine (5-FC) is a drug with low toxicity used to treat fungal infections. The cytosine deaminase enzyme from either yeast (yCD) or bacteria (bCD) converts 5-FC to the much more potent 5-FU.³ 5-FU is further processed by endogenous enzymes to one of three pyrimidine antimetabolites: 3-fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP) is a thymidylate synthase inhibitor, preventing deoxythymidine triphosphate synthesis, a precursor of DNA; 5-fluorouridine-5'-triphosphate (5'-FUTP) disrupts RNA processing by incorporating into RNA in the place of UTP and 5-fluorodeoxyuridine-5'-triphosphate (5-FdUTP) incorporates into and damages DNA.^{3,5–7}

The CD/5-FC system is capable of introducing a strong bystander effect, meaning surrounding cells will be affected, not just those that express the enzyme. 5-FC can freely diffuse between cells; it does not require direct cell-to-cell contact or gap junctional intercellular communication.^{7–9} This is an important advantage: *in vitro* experiments involving transfected cells expressing CD, grown together with untransfected cells, showed that the number of transfected cells can be as low as 1–30% and still produce enough 5-FU to inhibit growth of the other untransfected cells.^{8,10,11}

Herpes simplex virus thymidine kinase/ganciclovir prodrug system

The GDEPT system involving the enzyme herpes simplex virus thymidine kinase (HSVtk) and the prodrug 9-([2-hydroxy-1-(hydroxymethyl)methyl] guanine (ganciclovir or GCV) is currently the most extensively studied. GCV is phosphorylated to monophosphate GCV (GCV-MP) by the mammalian thymidine kinases but conversion is 1000 times faster with HSVtk.¹² This is the rate-limiting step and mammalian guanylate kinase then further phosphorylates GCV-MP to GCV-DP (diphosphate) and GCV-TP (triphosphate).¹³ GCV-TP causes cell death through the inhibition of cellular DNA polymerase and incorporation into DNA in the place of dGTP during cell division, resulting in single strand breaks in the DNA.^{14,15}

A drawback of the HSVtk/GCV system is that GCV-TP, being highly charged, is insoluble in lipid membranes. Therefore, the bystander effect will not occur through diffusion of GCV-TP into untransfected tumour cells. However, in preclinical studies transduction rates as low as 10% could still result in tumour regression.^{16,17} This is thought to be due to a combination of transfer of the toxic drug across gap junctions between cells, as well as the release of GCV-TP in apoptotic vesicles from dying cells and their subsequent phagocytosis by transfected cells.^{17,18} The immune system also plays a role in the bystander effect – administration of HSVtk/GCV treatment results in entry into the tumour of CD4⁺ and CD8⁺ T cells and macrophages and the heightened expression of the immune signalling molecules IL-2, IL-12, IFN- γ , TNF- α and GM-CSF.^{19,20}

Attempts have been made to improve the bystander effect of HSVtk/GCV. For example, various groups have worked on ways to increase expression of connexin proteins, which have a role in development of gap junctions, through the use of pharmacological agents or the co-expression of the protein with HSVtk.⁷ Other groups have constructed fusions between HSVtk and proteins that stimulate intercellular import and export, such as 8–11 amino acids from the human HIV-1 TAT protein.²¹ This fusion protein can move intercellularly, independent of gap junctions.

Other enzyme/prodrug systems

This review focuses on the two most commonly used GDEPT systems, CD/5-FC and HSVtk/GCV. However there are other systems which also have potential in the treatment of prostate cancer. For example, a phase I/II clinical trial involved the treatment of

localised prostate cancer with the GDEPT system nitroreductase/CB1954.²² Nitroreductases convert CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] to hydroxylamide and further processing produces an alkylating agent which can form DNA crosslinks independent of cell cycle.^{7,22} This GDEPT system results in a strong bystander effect as activated CB1954 can move freely through cell membranes.²³ Toxicity was mild and 6 out of 18 patients had an increase in time to PSA progression.

Cytochrome P450 enzymes are found in the liver and can be used in GDEPT to achieve local, targeted cell death, as well as a large bystander effect.⁷ They convert the oxazaphosphorine prodrugs (ifosfamide and cyclophosphamide) to 4-hydroxy-forms which are unstable and decay to phosphoramidate mustard and acrolein. These are alkylating agents that result in DNA crosslinks. Specific targeting of hypoxic cells has been investigated using the prodrug AQ4N which is metabolized to the cytotoxin AQ4 under hypoxic conditions.²⁴ GDEPT allows for the enhanced expression of cytochrome P450s in hypoxic cells to increase the level of AQ4N metabolism. Zhou et al. showed that if cytochrome P450-2C9 (CYP2C9) positive cells, H2C9, were co-cultured with the CYP2C9-negative prostate cancer cell line PPC-1 and treated with cyclophosphamide, the toxicity of the drug was four times higher than for PPC-1 cells alone, demonstrating a bystander effect.²⁵

The use of bacterial purine nucleoside phosphorylase (PNPs) as part of a GDEPT system is also under investigation. The enzyme converts adenosine analogues to cytotoxic adenosine analogues leading to disruption of ATP-dependent reactions. Cell kill can therefore occur in both proliferating and non-proliferating cells.^{7,26,27} There is a very strong bystander effect as the drugs are membrane permeable.²⁶ This GDEPT system has shown potential both *in vitro* and *in vivo*. For example, the PNP/6-methylpurine deoxyriboside system was demonstrated to be effective against PC3 xenografts in nude mice, resulting in tumour regression and increased survival.²⁸ Administration of the PNP gene to LNCaP-LN3 and PC3 xenografts in nude mice, alongside treatment with the prodrug fludarabine phosphate, resulted in an 100% increase in tumour doubling time and a greater than 50% inhibition of tumour growth.²⁹ In the TRAMP mouse model, in which the disease imitates human prostate cancer development, there was a significant decrease in tumour progression following PNP/fludarabine phosphate treatment.³⁰

GDEPT delivery vectors

Successful gene therapy treatment requires entry of the vector into the cells of interest. The most commonly used vector in gene therapy clinical trials is the adenovirus. As of June 2010, 23.8% of the 1644 trials undertaken involved this vector.¹³⁶ This review focuses on the adenovirus but there are multiple vectors, both viral and non-viral, that are being tested for use in GDEPT treatment. As well as the adenovirus, viral vectors include retrovirus, adeno-associated virus and herpes simplex virus. They have relatively high levels of gene delivery but there are issues with patient safety. Also, it can be hard to manufacture these vectors on a large scale.⁴ Non-viral methods of gene delivery include using liposomes, nanoparticles, insertion of naked DNA and insertion of gold particle coated DNA using gene guns.^{3,4,31} Large scale production is easier for non-viral vectors. They are less likely to cause an immune response compared to viral vectors but they are also less efficient at gene transfer and result in only transient expression.^{4,32} The vector chosen depends on the nature of the disease being targeted. In the case of cancer, only short-term expression is required, compared to corrective gene therapy.²⁷ More specifically, the behaviour of the tumour is important. Prostate tumours are relatively slow growing so require a vector that can transduce both

non-dividing and dividing cells.²⁷ The adenovirus meets both these criteria.

Optimal adenoviral delivery

Finding the most favourable route for adenoviral delivery is important to the success of GDEPT. This was examined in a canine model through the comparison of adenoviral transduction efficiency following its administration by three different routes: intravenous, intra-arterial and intraprostatic injections. The vector expressed β -galactosidase and it was found that levels of this enzyme in the prostate were highest after intraprostatic injection.³³ De Weese et al. adapted techniques used for brachytherapy to administer the oncolytic adenovirus CV706 for the treatment of locally recurrent prostate cancer.³⁴ They used a computerised planning system to determine the best locations for stereotactic intraprostatic injection. A similar method was used by Barton et al. to determine the treatment design for two prostate cancer patients, based on their previous findings for gene expression distribution for an oncolytic adenovirus in naïve canine prostate and human tumour xenografts.³⁵

Transcriptional control

Most of the clinical trials for GDEPT treatment of prostate cancer have involved the use of non-specific promoters to achieve high levels of enzyme gene expression. Tissue/tumour-specific activation of the prodrug can be achieved through the use of promoters that restrict expression of the enzyme to target cells. This approach should mean a reduction in side effects as it lowers toxicity to healthy tissues.^{27,36} In the case of prostate cancer, the tissue-specific PSA gene promoter has been utilised, which can be coupled with the PSA enhancer to further improve specificity.^{37,38} The potential of the prostate-specific membrane (PSMA) promoter has also been investigated^{39,40} as has the probasin promoter.⁴¹ For the treatment of metastatic prostate cancer, the promoter for the osteocalcin bone protein has been used to control expression of HSVtk and has reached phase I clinical trial.⁴² The strategy for targeted therapy means it is possible to restrict expression of the enzyme transgene to specific microenvironments, such as areas of hypoxia within the tumour. This is useful as hypoxic cells are more resistant to both radiation and chemotherapy.^{36,43} The use of hypoxia response elements from various genes have been tested as a means of achieving hypoxia-specific expression.^{44,45}

Combining GDEPT with radiation therapy

Together with the use of the biomarker prostate specific antigen (PSA), which has allowed for earlier detection of prostate cancer, technological advances in radiation therapy have led to better treatment results.⁴⁶ Progress in technology has allowed for improved tumour targeting, together with escalated doses of radiation to enable increased cell kill.^{47,48} However, the higher levels of tumour destruction achieved by increasing radiation levels must be balanced with the risk of concurrent damage to normal tissue. In addition, subpopulations of clonogenic tumour cells may be radioresistant despite high dose levels, leading to local recurrence and/or formation of metastases in a proportion of the patients.⁴⁷ As a consequence there is a clear requirement for the exploration of additional avenues to further improve the efficacy of radiation therapy.

Specific characteristics of prostate cancer make it a particularly suitable candidate for GDEPT. Although loss of function affects quality of life of the patient, the organ is not crucial for survival. Its location makes it relatively easy to access for direct intratumoural

treatment strategies: specific drugs can be given through a transrectal, transperineal or transurethral route and biopsies can be taken to monitor progress. Prostate tumours express a highly specific protein, PSA, a marker for the disease that is extremely useful in assessing the success of the therapy. Existing phase I/II trials suggest that injections of the vector, as well as administration of the prodrug and follow-up monitoring, can all take place in an outpatient setting, reducing the potential cost of treatment.^{32,49} There are nonetheless difficulties in treating prostate cancer using gene therapy. Its multifocal growth pattern makes it challenging to target cancer cells and means that delivery of the vector to the whole gland is necessary. In addition, heterogeneity within the tumour cell micro-environment and intrinsic variations in genetic mutations causing the disease mean it can be hard to develop a therapy which will effectively kill all tumour cells.^{32,49}

Gene therapy has the potential to improve the outcome of radiation therapy through a number of mechanisms. One potential benefit of combining the two treatment strategies is an increase in the radiosensitivity of the tumour. This would allow for a higher level of tumour cell death with lower doses, reducing toxic side effects. In the case of the CD/5-FC system there is evidence that 5-FU is a moderate radiosensitizer.⁵⁰ As mentioned, it is processed to one of three pyrimidine antimetabolites, one of which, 5-FdUMP, is thought to be involved in radiosensitization as it prevents DNA repair following irradiation.^{50,51} Also, gene therapy is effective during the S phase of the cell cycle when DNA synthesis is taking place. This is the stage when cells are normally more resistant to radiation therapy.⁴⁷

In the HSVtk/GCV model, phosphorylated GCV acts as a radiosensitizer as the nucleotide analogue prevents DNA replication and thereby stops the repair of DNA damage caused by radiation.⁵² Of note, the combination of HSVtk and GCV with radiation has been shown to enhance treatment efficacy for prostate cancer *in vivo*.⁵² In the study by Chhikara et al., a significant rise in the inhibition of tumour growth and increase in survival times in a RM-1 prostate cancer mouse model was observed when the therapies were united, compared to radiation or gene therapy alone.

It has been demonstrated that radiation treatment increases the level of uptake of the adenovirus into mammalian cells as well as tumour xenografts.^{53–56} Stevens et al. showed that irradiation of mammalian cells with up to 7 Gy improved subsequent adenoviral transduction by up to 40-fold in the human neoplastic cell line A549.⁵⁵ Notably, the effect was dose dependent. Expression of the transgene, *lacZ*, lasted longer in irradiated cells and this was shown to be secondary to the integration of the adenovirus into the host DNA. This phenomenon was also observed by Zhang et al. following exposure of mammalian cell lines and rat hepatocytes to 4 Gy of radiation prior to transduction with a green fluorescent protein (GFP) expressing adenoviral vector – there was an associated 27.1-fold increase in GFP expression.⁵⁶ *In vivo*, the level of transgene expression (this time *lacZ*) in rat intrahepatic colon carcinoma xenografts was also raised by combining it with radiation (25 Gy).

The same group went on to show the importance of Dynamin 2, a membrane-associated protein, in this process. In cells treated with an antibody to Dynamin 2, the level of viral uptake and gene transfer in response to radiation was significantly reduced in rat hepatocytes and human colon carcinoma cells.⁵⁴ In contrast, antibodies for coxsackie B virus and adenovirus receptor (CAR) or integrin α_v had little or no effect. mRNA and protein levels of Dynamin 2 were shown to rise after irradiation. The introduction of siRNA specific to Dynamin 2 led to a significant drop in the amount of viral uptake and gene transfer in radiation treated cells. In colon cancer xenografts, Dynamin 2 levels were higher following irradiation, as was the amount of viral uptake.⁵⁴ The results of this study point to an important role for Dynamin 2 in the radiation-induced rise in

adenoviral uptake. Egami et al. demonstrated that in pancreatic tumour cells, as well as subcutaneous tumours in nude mice, transgene expression levels following infection with an adenoviral vector were higher when radiation was also administered.⁵³ Combining adenovirus administration with radiation therapy has the potential to improve transgene expression levels, thereby increasing the efficacy of GDEPT.

Clinical trials of GDEPT in combination with radiation therapy

GDEPT for the management of local failure following radiation therapy

A subset of men treated with radiation therapy will experience treatment failure and recurrence of the disease. Once biochemical failure has been established, a non-curative follow-up treatment option is salvage androgen suppression therapy. This is achieved in most cases through the administration of luteinizing hormone-releasing hormone (LHRH) agonists, possibly combined with an anti-androgen.⁵⁷ Side effects resulting from androgen deprivation therapy include hot flushes, lack of libido, gynecomastia, cognitive dysfunction and depression and over time patients can develop osteoporosis and anaemia and longer term risk of accelerated ischaemic heart disease.⁵⁸

Curative treatment strategies used after radiation therapy are uncommon but include salvage radical prostatectomy. This treatment results in a cancer-specific survival rate at 10 years of 65–73% in highly selected patients but the side effects are significant, due in part to the difficult nature of the surgery following radiation-induced damage to the pelvic tissues.^{59–63} The side effects include erectile dysfunction and incontinence.^{59,60} Salvage cryoablation is an alternative investigational method which allows for destruction of locally recurrent tumours. Of note, the initial patients treated in this way frequently suffered side effects. For example, Bales et al. were able to achieve negative biopsy in 86% of their patients at 3 months post-cryoablation but 95% of the patients suffered from incontinence.⁶⁴ However, the technique has been developed and improved since then, moving away from using liquid nitrogen cryoprobes to argon/helium gas.⁶⁵ Ghafar et al. attained biochemical disease-free survival in 86% and 74% of patients at one and two years respectively using argon gas cryoablation and the level of incontinence was 7.9%.⁶⁶

In patients where the tumour remains localised another option is salvage brachytherapy which involves the administration of further higher doses of radiation through the use of radioactive seeds or catheters.⁶⁷ Recently, four year biochemical disease-free survival rates of 70% and 75% have been obtained using this technique, with the most common side effects being gastrointestinal and genitourinary toxicity.^{68,69} Finally, some groups have used high intensity focused ultrasound (HIFU) as a method of salvage therapy following RT.^{70–72} Ultrasound energy is targeted to a specific area causing the temperature to increase to more than 60 °C and consequently resulting in cell death.⁷⁰ There are limited data on the efficacy of HIFU in the context of salvage therapy. In the largest study to date, 3 year progression-free survival rates were 53%, 42% and 25% for low, intermediate and high-risk patients respectively, although there was a high level (49.5%) of urinary incontinence in the patient cohort.⁷¹

Despite these advances, none of the current salvage treatments is ideal. GDEPT was thus initially investigated for its potential for the treatment of locally recurrent prostate cancer to increase PSA doubling time and potentially delay the time to initiation of androgen ablation. Table 1 summarises the results of the clinical trials involving GDEPT for the treatment of prostate cancer. The first phase I clinical trial to demonstrate the potential benefit of GDEPT in locally recurrent prostate cancer was carried out in Baylor

College of Medicine, Houston in 1999.⁴⁹ Selected patients had local recurrence of prostate cancer, as demonstrated by biopsy, at least one year after definitive irradiation therapy, along with a rising PSA level but no evidence of metastasis of the tumour. Herman et al. used a replication-deficient adenovirus to transfect patients with HSVtk. A series of escalating concentrations of the adenovirus were administered to patients intraprostatically followed by intravenous injection of GCV two weeks later. Administration of the therapy resulted in a decline of more than 50% in the serum PSA level of 3 of the 18 patients. For the patient at the highest dose level this lasted for more than a year. Moreover, the safety of this treatment was established, with the majority of patients displaying minimal toxicity or none at all, although one patient who received the highest dose of the adenovirus vector (1×10^{11} infectious units) experienced grade 4 thrombocytopenia and grade 3 hepatotoxicity.

This study led to an extended phase I/II trial using the ADV/HSVtk adenovirus to treat 36 patients with clinically localised recurrent prostate cancer at least 18 months after radiotherapy and increasing serum PSA levels, including the 18 patients from the previous trial.⁷³ Twenty-seven of the patients showed a drop in serum PSA after vector infection and administration of GCV, with a mean reduction of 28%. The average time taken for PSA levels to return to their original value was calculated at 8 months. Treatment effect on PSA doubling time (PSADT) was examined in 22 of the patients (those who had not been excluded due to insufficient data or the development of metastatic disease), with a reported increase in PSADT from 15.9 months to 42.5 after gene therapy ($p = 0.0271$). PSADT has been shown to have strong prognostic power and is predictive of metastasis formation and prostate cancer-specific mortality.^{74–77}

In separate studies, oncolytic replication-competent adenoviruses have also been investigated for the treatment of localised prostate cancer. The first clinical trial involving a prostate-specific oncolytic adenovirus was set up by investigators at John Hopkins Medical Institute in 2001.³⁴ The adenovirus was CV706, originally reported by Rodriguez et al., a replication-competent, E3-deleted Ad5 adenovirus that contains a promoter-enhancer construct from the human PSA gene, thereby restricting replication to PSA-expressing cells.⁷⁸ All patients chosen had locally recurrent prostate cancer following EBRT, as well as increasing PSA levels. Treatment was found to be safe with low toxicity – most men experienced only mild adverse events (AEs). Grade 3 toxicities did occur in five men but these were all reversible and were not deemed to be directly related to the drug itself. In 65% of patients, PSA dropped by $\geq 30\%$ compared to levels pre-treatment. Notably, 25% of patients showed an even greater reduction ($\geq 50\%$) in PSA, all of whom had received the highest or second highest dose of CV706, suggesting the response was dose dependent. Evidence for *in vivo* adenovirus replication was also seen as there was a secondary peak in levels of CV706 DNA in the circulation of most patients approximately 3 days after treatment.

A logical progression for this cancer gene therapy approach was to combine the oncolytic adenovirus with GDEPT. Conceptually, the use of a replication-competent adenovirus would result in higher levels of gene expression and increase the spread of the virus into nearby tumour cells.⁷⁹ This was first tested in a Phase 1 study by Freytag et al. in which the adenovirus (Ad5-CD/TKrep) was used to deliver a CD/HSVtk fusion gene.⁸⁰ The expression of these genes was expected to enable the activation of non-toxic prodrugs to their toxic forms. Furthermore, both suicide gene systems (CD/5-FC and HSVtk/GCV) increase sensitivity to radiation in addition to the oncolytic properties of the adenovirus. The strategy was tested in patients who had evidence of recurrent prostate cancer at least 1 year after definitive radiation therapy, as well as rising serum PSA levels (but <20 ng/ml) and an absence of metastatic

Table 1
GDEPT trials for the treatment of prostate cancer. Summary of clinical trials that used GDEPT to treat localised, locally recurrent and metastatic prostate cancer, including the toxicity and the outcome of the therapy.

	Vector/ Enzyme + prodrug(s)	Phase	N	Toxicity	Results	References
Newly diagnosed localised prostate cancer	ADV/HSV-tk + vGCV	I/II	30	Most AEs grade 1 or 2. Grade 3 ALT elevation in one patient. Grade 3 acute genitourinary toxicity in one patient	The clinical outcome of the treatment was assessed at a later date ⁸⁴	83
	ADV/HSV-tk + vGCV	I/II	59		Biochemical control in all low and intermediate-risk patients, as well as negative biopsies. Biochemical failure and metastases in 3 (75%) of the high-risk patients	84
	Ad5-CD/TKrep + 5-FC + vGCV	I	15	Most AEs grade 1 (65%) or grade 2 (29%)	Mean PSA half-life shorter with prodrug therapy (>1 week) than for 3D-CRT alone (0.6 vs. 2.4 months)	2
	Ad5-yCD/ mutTKSR39rep-ADP + 5-FC + vGCV	I	9	Most AEs grade 1 or 2 (92%)	(Results combined with earlier trial ²) 0 of 12 intermediate-risk patients had positive biopsies. Significantly different from the expected result of ≥30% for radiation therapy alone	85
Locally recurrent prostate cancer	ADV/HSV-tk + GCV	I	18	Mild toxicity in 4 patients (grade 1–2). Significant toxicity in 1 patient at highest dose	Drop in serum PSA levels of 50% or more in three patients. Lasted up to 1 year	49
	ADV/HSV-tk + GCV	I/II	36	Mild toxicity in 13 patients (grade 1–2). Significant toxicity in two patients at highest dose (grade 3–4)	Drop in serum PSA levels in 27 patients (mean 28%) PSADT increased from 14.3–42.5 months	73
	Ad5-CD/TKrep + 5-FC + GCV	I	16	Most AEs grade 1 (82%) or grade 2 (12%)	Drop in serum PSA levels of ≥25% in seven patients (44%) and ≥50% in three patients (19%) 5-year follow-up: PSADT improved from a mean of 17–31 months Delay in the initiation of androgen suppression therapy (average 2.1 years)	80 81
Metastatic prostate cancer	Ad-OC-hsv-TK + vACV	I	11	Most AEs grade 1 or 2. Grade 3 lymphopenia in two patients. Grade 3 neutropenia in one patient	Increase in cell death in the treated lesions of 7 patients. One patient had a decrease of >50% in the level of PSA	42
	Ad-OC-hsv-TK + VAL	I/II	6	Most AEs grade 1 or 2. Grade 3 lymphopenia in three patients	Decline in PSA (318.3–4.9 ng/ml) in 1 patient with time to PSA progression of 1 year (Chemotherapy given to three patients following failure of GDEPT and was effective in all 3)	92

3D-CRT, 3D-conformal radiation; 5-FC, 5-fluorocytosine; Ad5, adenovirus type 5; AEs, adverse events; ADP, adenovirus death protein; ADV, adenovirus; CD, cytosine deaminase; GCV, ganciclovir; HSV-tk, herpes simplex virus thymidine kinase; OC, osteocalcin; PSA, prostate specific antigen; PSADT, prostate specific antigen doubling time; rep, replication competent; vACV, valacyclovir; VAL vGCV, valganciclovir; yCD, yeast cytosine deaminase.

disease. Sixteen patients, in four groups, were given escalating doses of the virus up to 10¹² virus particles. Two days subsequent, patients began 1–2 weeks of 5-FC and GCV therapy. Toxicity was low, with most of the AEs classed as grade 1 (82%) or grade 2 (12%). Only one AE, a grade 1 thrombocytopenia, necessitated a change to the treatment plan. Of the 6% of AEs that were grade 3, all were self-limiting. Of the sixteen patients in the trial, 7 (44%) showed a drop in serum PSA levels of ≥25% and 3 (19%) showed a ≥50% decrease for at least 4 weeks. The longest duration of PSA reduction was 4 months. In most cases the reduction in PSA levels was greatest during 5-FC/GCV therapy, followed by a leveling off of PSA levels or a much slower decrease until the virus was no longer present in the prostate, indicated by an absence of Ad5-CD/TKrep DNA in the blood. Therefore, Freytag et al. concluded that the majority of antitumour activity was a result of the suicide gene systems and that the subsequent, less dramatic, decrease in PSA represents the oncolytic activity of the adenovirus. The success of the therapy was also assessed using prostate needle biopsies after 2 weeks where coagulative necrosis, indicating tumour destruction, was seen in several cases, with two patients remaining negative for adenocarcinoma one year after therapy.

Recently, a five year follow up has been carried out by Freytag et al. on their trial of the replication-complement adenovirus, Ad5-CD/TKrep.^{80,81} As mentioned previously, the decrease in serum PSA levels was brief (<6 months). However the results from the follow-up study are promising. No long term side-effects were observed. PSADT was used as a measure of the effectiveness of the therapy. Of the sixteen original participants, fourteen were

evaluable. It was found that PSADT improved from a mean of 17–31 months (median 16–22 months; *p* = 0.014) following treatment. It is unclear why the PSADT continues to increase, even in the absence of the adenovirus; Freytag et al. suggest anti-tumour immunity may have been contributory. In support of this, previous studies with mice models have shown that CD and HSVtk suicide gene therapy results in T cell-dependent antitumour immunity.^{20,82} Given the possible activation of an immune response, Freytag et al. believe it suggests this therapy may have the potential to be used to treat metastatic as well as local disease. The long term therapeutic effect of the treatment was also assessed by calculating the extent of the delay in the initiation of salvage androgen suppression therapy and was an average of 2.1 years.

In summary, the results of the clinical trials examining the benefit of GDEPT in localised recurrent prostate cancer are encouraging. The safety profile has been established and the benefit to patients has also been demonstrated. GDEPT could therefore be an option in follow-up treatment if the initial therapy fails. An alternative therapy that could delay or even replace androgen suppression therapy for patients with rising PSA levels following definitive therapy could greatly improve quality of life.

GDEPT to improve radiosensitivity in newly diagnosed localised prostate cancer

The success of treatment for localised prostate cancer is highly variable. Although rates of long term disease-free survival are high for low-risk prostate cancer following radiation therapy (~80%),

they are much lower for those who have intermediate or high-risk forms of the disease (25–75%).² Therefore, ways to improve the initial therapy for localised prostate cancer and prevent metastatic tumour formation are necessary. The potential of GDEPT has been examined in the case of newly diagnosed tumours.

The first clinical trial to investigate the potential of combining radiation therapy with GDEPT was performed by Teh et al.⁸³ The phase I/II study involved 30 patients divided between three arms: Arm A consisted of 13 low-risk patients (PSA <10 ng/ml, Gleason score <7, Stage T1–T2a); the 14 patients in Arm B were high-risk (PSA ≥10 ng/ml, Gleason score ≥7, Stage T2b–T3) and the 3 Arm C patients had Stage D1 cancer and therefore had positive pelvic lymph nodes. The patients were treated with a combination of ADV/HSVtk by intraprostatic injection and the prodrug valacyclovir given 24 h later and for 14 days, together with a mean dose of 76 Gy of radiation given by intensity-modulated radiation therapy (IMRT). In addition, Arm C patients received 45 Gy to the pelvic lymphatics and those in Arms B and C were treated with androgen deprivation therapy. The initial aim was to assess the safety of the treatment regime. Toxicity levels were found to be low and there was no increase in the amount of genitourinary or gastrointestinal injury compared to IMRT alone. Most AEs were grade 1 or 2. One patient experienced a grade 3 rise in the liver enzyme ALT but this returned to normal without intervention.

The results of an extended trial and follow-up of patients were later published by Teh et al.⁸⁴ In the study, PSA and biopsy results for 59 patients divided between Arm A (29 patients), Arm B (26 patients) and Arm C (4 patients) were reported. PSA measurements showed biochemical control had been achieved in all patients in Arms A and B (median follow-up 13.4 and 13.9 months respectively). Three of the four patients in Arm C, however, experienced biochemical failure. The disease had metastasised to the bones in two of these men and to the para-aortic lymph nodes in the other. For arm A and B, the biopsy results showed an increase in the carcinoma-negative samples over 24 months. The group recognised there was a chance that the biopsies failed to detect tumour and that more long-term follow-up was needed but the initial results of the trial were positive.

Freytag et al. further investigated the Ad5-CD/TKrep gene therapy treatment and examined its effects in combination with three-dimensional conformal radiation therapy (3D-CRT) therapy in patients with newly diagnosed intermediate to high-risk prostate cancer at stage T1c to T3.² The mean Gleason score was 8, with a range of 6–9. The mean serum PSA was 12.9 ng/ml, with a range of 2.5–30.9 ng/ml. Patients had no evidence of metastatic disease, as determined by bone scan and CT scan of the abdomen and pelvis. In this study, Ad5-CD/TKrep was administered to 15 patients by intraprostatic injection. This was followed two days later, with the administration of 5-FC and valganciclovir (vGCV, an orally administered alternative to ganciclovir), as well as 70–74 Gy of radiation therapy for 1, 2 or 3 weeks. In keeping with the previous trial, AEs were predominantly mild (65% grade 1) or moderate (29% grade 2). The common side effects of 3D-CRT, bladder and bowel toxicities, were no more frequent in this trial, despite the inclusion of an oncolytic adenovirus and gene therapy that increases radiosensitivity. Transgene expression in the prostate lasted up to 3 weeks after injection. The mean PSA half-life for those receiving prodrug therapy for greater than 1 week was shorter than for 3D-CRT alone (0.6 vs. 2.4 months).

Freytag et al. continued their work by developing an improved, second-generation adenovirus and testing it in combination with IMRT in men with newly diagnosed prostate cancer.⁸⁵ The new adenovirus, Ad5-yCD/mutTK_{SR39}rep-ADP, contains the yeast, rather than the bacterial CD gene and the mutant gene SR39 HSVtk, both of which produce more catalytically active enzymes. Experimental data suggested an increase in radiosensitivity and

chemotherapeutic effect using this adenovirus. In addition, the vector codes for the adenovirus death protein (ADP), which improves cell killing and spread of the virus. In a phase I trial, nine patients received 10^{11} , 10^{12} or two doses of 10^{12} virus particles of the second generation adenovirus. Three days after each injection, they began a 13 day course of 5-FC, vGCV and 74 Gy IMRT. There was no increase in toxicity compared to the original first generation adenovirus, in spite of an increase in catalytic activity. To assess the merit of the combination of suicide gene therapy with radiotherapy, the result from the trials of Ad5-CD/TKrep and Ad5-yCD/mutTK_{SR39}rep-ADP were merged and compared to the expected results for radiotherapy alone. In ≥40% of the patients, one would expect a positive prostate biopsy following radiation therapy. However, in the case of these trials, in which radiotherapy was used alongside suicide gene therapy, only 22% of the evaluable patients had a positive final biopsy ($p = 0.038$). The results were more informative when the patients were divided by prognostic risk category: none of the 12 intermediate risk patients was positive for adenocarcinoma at biopsy, which is significantly different from the expected result of ≥30% ($p < 0.01$). On the other hand, the result for the high risk group (45%) was not significantly different from the expected level using radiotherapy alone (56%) ($p = 0.72$).

An adaptive seamless randomised phase II/III trial to follow-up these results has been approved and the group are presently recruiting patients.⁸⁶ 280 patients will be divided into two arms. Arm one receive one dose of 10^{12} virus particles of Ad5-yCD/mutTK_{SR39}rep-ADP intraprostatically, followed by a two week course of 5-FC, vGCV alongside an eight week course of 80 Gy IMRT. Those in Arm two receive IMRT alone. The Phase II component of the trial will involve assessment of toxicity in the first 21 patients in Arm 1, 90 days into the trial. Efficacy will be measured using time free from biochemical and/or clinical failure as well as prostate biopsies at year 2 to assess local tumour control and analysis of overall survival rates. It will be the first randomized controlled trial using gene therapy to treat non-metastatic prostate cancer.

The results of previous trials suggest that GDEPT can be safely used alongside radiation therapy and when combined, it has the potential to improve treatment outcome compared to radiation therapy alone. It is encouraging that this treatment is now moving into a Phase II/III trial.

GDEPT for the treatment of metastatic prostate cancer

In a proportion of men, either due to late diagnosis or treatment failure, prostate cancer will become androgen independent and metastasise. Most tumours initially metastasise to bone, but distant lymph nodes, the lungs and the liver may become involved. Osseous metastases are found in about 90% of those with advanced disease.^{87,88} Currently docetaxel-based chemotherapy for metastatic disease improves bone pain and quality of life,⁸⁹ however its use is predominantly palliative with a modest gain in long-term survival.⁷⁹ A limited number of studies have investigated the potential of GDEPT to improve treatment of metastatic disease.

Kubo et al. carried out a phase I clinical trial using osteocalcin promoter-driven HSVtk (Ad-OC-hsv-TK).⁴² Osteocalcin is a noncollagenous bone protein. Its expression is upregulated in bone metastatic prostate cancer and the osteoblast cells at the site.^{90,91} The group hypothesised that this property would enable Ad-OC-hsv-TK to target the tumour-associated stroma cells as well as the tumour itself. The trial involved 11 patients – two with local recurrence of cancer after surgery, four with lymph node metastases and five with osseous metastases. The men had previously been treated with surgery, radiation therapy, chemotherapy and/or hormonal therapy. Each patient received two intralesional injections of Ad-OC-hsv-TK at one of three dose levels (5×10^9 , 5×10^{10} and

5×10^{11} particles/injection) and 1 g of valacyclovir taken orally every day for 21 days. The treatment was shown to be relatively safe with most of the AEs at grade 1 or 2. Using TUNEL staining, an increase in cell death was seen in the treated lesions in seven of the patients (63.6%). In one patient, the treated lesion was stabilized for 317 days, whilst another patient experienced a decrease of >50% in PSA level. The remaining patients did not show a significant fall in PSA or reduction in tumour size.

This study led to a phase I/II clinical trial of Ad-OC-hsv-TK / VAL gene therapy.⁹² Six patients were involved, all of whom had bone metastases and relatively high PSA levels (46.6–455.5 ng/ml). The patients had been treated with hormone therapy and chemotherapy already, as well as radical prostatectomy and/or radiation therapy in some cases. Once again the safety of the treatment was demonstrated. One of the 6 patients had a distinct decline in PSA (318.3–4.9 ng/ml) and time to PSA progression was one year. CT scans of this patient showed a decrease in the metastatic density of the lesion at day 180. Following PSA failure after gene therapy treatment docetaxel and estramustine combination chemotherapy was administered to three of the patients. This was effective in all three, with time to PSA progression at 21, 7 and 4 months.

In this limited range of trials, the relative safety of GDEPT in patients with metastatic prostate cancer was demonstrated. In addition, there have been some promising results in terms of the benefit of the treatment to the patients. Currently, available therapies are merely palliative so any improvement in this is desirable.

Future development of GDEPT strategies

For successful transition of gene therapy to the clinic, efficient and effective methods of gene delivery will be necessary, as well as ways to monitor transgene expression to assess the efficacy of the therapy. Several strategies have been used to achieve these goals and are discussed below.

Improved delivery

As previously mentioned, adenoviruses remain the most widely used vector in gene therapy clinical trials. Entry of the virus occurs by endocytosis, when fibre proteins found on all 12 vertices of its icosahedral capsid interact with receptors on the cell surface.^{93,94} Of the serotypes of adenovirus, the most commonly investigated is Ad type 5 (Ad5) of subgroup C.⁹⁵ Cell entry by this subgroup, through binding of the vector, has been shown to be initially dependent on cell surface expression of CAR.^{96,97} This is accompanied by an interaction between the penton base of the fibre protein with the vitronectin receptors, integrins $\alpha_1\beta_3$ and $\alpha_1\beta_5$, on the cell surface. Although the presence of the vitronectin receptors is not necessary, it greatly improves entry efficiency. Together, these interactions lead to viral entry.^{96,97}

An improvement in the transduction efficiency of adenoviral vectors is desirable. The increase in vector uptake when radiation therapy is combined with vector administration has been previously mentioned. Other ways to improve delivery are also being investigated. There are a number of issues that need to be addressed. Many cell types do not, in fact, express CAR at high levels, including skeletal and smooth muscle cells, dendritic cells and haematopoietic stem cells.⁹⁸ The low degree of expression means the dose of vector necessary is large, leading to a higher number of side effects. Although CAR may not be present on the cells of interest, its expression in general is widespread, as is that of the secondary and third receptors of Ad.⁹⁸ This presents another problem – non-specific entry of the vector causing toxicity. In the case of prostate cancer, Rauen et al. used a polyclonal antibody against human CAR to examine its levels in normal prostate tissue as well as primary

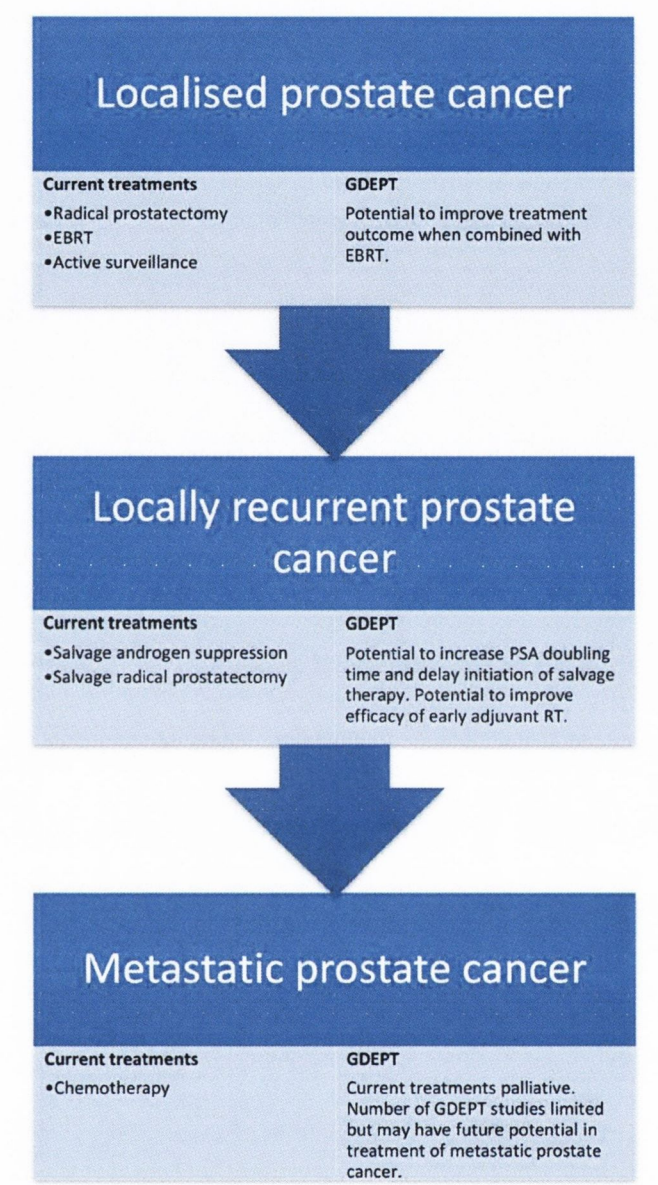


Fig. 1. Clinical potential of GDEPT at various stages of prostate cancer progression.

and metastatic tumour samples.⁹⁹ Examination of paraffin-embedded tissue showed that expression levels of CAR were high in the luminal and lateral cell membrane of normal prostate samples. However, the number of cells expressing CAR, as well as the strength of the expression, decreased with increasing Gleason grade for the prostate tumour samples. Metastatic samples were found to have high levels of CAR expression. Therefore the Gleason Grade and stage of cancer growth may be an important factor in determining the degree of successful viral delivery.

A number of approaches to overcome these problems have been investigated, through the manipulation of the adenovirus to create a more efficient, targeted delivery system (Fig. 2; discussed in detail by Campos et al.¹⁰⁰). One approach is to genetically alter the capsid of the virus to direct it to particular cell types and allow attachment of the fibre proteins to other receptors on the cell surface. In this strategy, specific peptides are inserted into the fibre knob. For example, in one study poly-lysine residues (K7 peptide) were inserted into the C-terminus and in a different study an

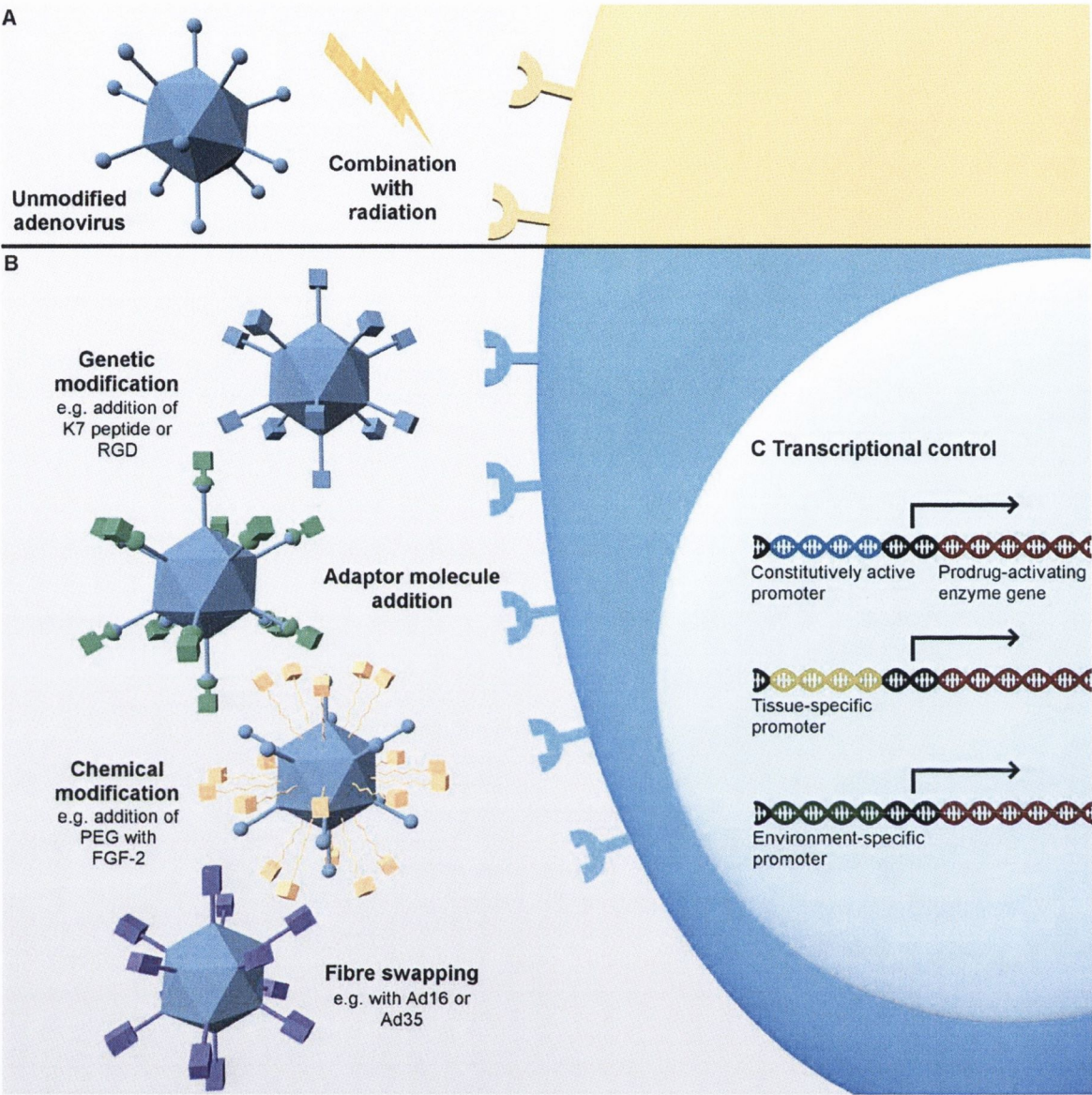


Fig. 2. Transductional and transcriptional targeting of GDEPT. (A) Radiation has been shown to increase the level of uptake of the adenovirus into mammalian cells and tumour xenografts. (B) Manipulation of the adenovirus capsid allows it to bind to receptors other than CAR. This can be achieved through genetic modification, adaptor molecule addition, chemical modification or the replacement of the fibres with those from alternative CAR-independent members of the adenovirus family. (C) The use of specific promoters allows for tissue/tumour targeting of expression of the prodrug activating enzyme and therefore targeted cell death. This may reduce the risk of toxic side effects.

Arg-Gly-Asp (RGD) peptide was inserted into the HI loop.^{101,102} This increased the tropism of these vectors and allowed them to enter cells not normally susceptible to transduction. Other approaches have involved modification of different parts of the viral capsid, including the hypervariable region 5 (HVR5) loop of the hexon, the C-terminus of protein IX and the penton base.^{103–105}

Another way to manipulate vector targeting involves the use of bispecific or bifunctional adaptor molecules, a capsid-binding domain joined to a cell-binding ligand such as fibroblast growth factor-2 or epidermal growth factor.^{106,107} However, this technique is limited by batch-to-batch variation in the preparation of the fusion proteins and the weak non-covalent protein–protein binding of the molecules to the capsid, which may be unable to compete *in vivo*.¹⁰⁰

Chemical modification of the capsid is another option for adapting adenovirus entry. One chemical used is polyethylene glycol

(PEG) which has the advantage of lengthening the time the vector is present in the blood, stopping neutralisation by antibodies as well as lessening the innate immune response to the vectors.^{108–110}

However this modification of the vector also results in decreased infectivity. This can be counteracted through the attachment of peptides like FGF-2 to PEG, improving transgene expression and targeting.¹¹¹ Alternatives to PEGylation include addition of poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA) to the viral surface.¹¹²

Attempts to overcome the requirement of adenovirus type 5 (Ad5) for CAR to bind to the cell surface have taken advantage of the variety in the adenoviridae family.¹⁰⁰ Tropism of the vector can be altered through the swapping of fibres of the Ad5 with those of members of subgroup B to create fibre-chimeric vectors. Examples include Ad16 and Ad35.^{95,98,113,114} Unlike subgroups A, C, D, E and F, most of whose members need CAR, subgroup B uses other

receptors: CD46, CD80/CD86 and “receptor X”.^{115–120} Therefore, the vectors become CAR independent, allowing high levels of transduction of a wide range of cell types.

Non-invasive imaging of transgene activity

The ability to image transgene activity is crucial for the progress of GDEPT. Research is being carried out to develop non-invasive methods of monitoring transgene expression to help assess the success of the treatment.

Liu et al. describe their continuing work on examining transgene expression with the reporter gene *lacZ*.¹²¹ This gene encodes β -galactosidase (β -gal), the activity of which is detected using a substrate of the enzyme, 2-fluoro-4-nitrophenyl β -D-galactopyranoside (OFPNPG). Using ¹⁹F nuclear magnetic resonance, the cleavage of OFPNPG can be detected. This was tested in *lacZ*-expressing PC3 prostate tumour xenografts in mice. This work describes a novel method for confirming transgene expression with possible application in GDEPT. Continued investigation is being carried out to develop reporters with lower toxicity.

A number of studies have examined the potential for imaging of transgene activity using a single gene as the reporter as well as the therapeutic gene. One of the most recent examples of this was research published by Gade et al. which used magnetic resonance spectroscopy imaging (MRSI) to examine the *in vivo* expression of the fusion gene encoding cytosine deaminase-uracil phosphoribosyltransferase (CD-UPRT) in CD-UPRT⁺ and CD-UPRT⁻ W256 xenografts in nude mice.¹²² The prodrug 5-FC is converted to 5-FU by CD, then to FUMP by UPRT. This is detected by ¹⁹F MRSI. In mixed tumours (consisting of both wild type and CD-UPRT⁺ W256 cells) this technique was able to detect heterogeneity in enzyme activity.

A second reporter gene system involves HSVtk and uses the therapeutic gene itself. HSVtk phosphorylates certain labelled probes, such as the acycloguanosines (e.g. ganciclovir, penciclovir), preventing movement of the compounds back across the cell membrane, thereby trapping them and allowing their visualisation using positron emission tomography (PET).^{123–126} Therefore, using a suitable substrate, the localisation and magnitude of HSVtk gene expression can be assessed. To enhance efficacy of the enzyme as a reporter, the mutant gene HSV1-sr39tk was developed, the product of which can more efficiently act on fluorinated acycloguanosine substrates, increasing the sensitivity of the PET reporter system.¹²⁴ Importantly an improvement in enzyme activity on acycloguanosines means an increase in cytotoxicity and more effective GDEPT.^{124,125}

An alternative reporter gene system that uses nuclear medicine imaging involves a mutated version of the dopamine type 2 receptor (D2R) gene, which can bind to its ligand but is incapable of activating downstream signalling. Using this system and the radiolabelled probe ¹⁸F-fluoroethylspiperone (FESP), *in vivo* and *in vitro* assays have shown that measurement of FESP build up using PET is a true indicator of gene expression in the tissues of interest.^{127,128} In work by Kummer et al., tests were carried out using a universal HSV amplicon vector that expressed both HSVtk and D2R in a bicistronic cassette in a human glioma model. The PET studies demonstrated that the D2R system gave as much information on gene expression *in vivo* as the HSVtk gene system.¹²⁹

The reporter gene systems (HSVtk and D2R) that use PET can be expensive and complex.¹²⁵ Therefore, some systems have been developed that require a γ -camera instead of a costly PET unit; examples are the somatostatin reporter (SSTR) 2 gene and ¹¹¹Inoctreotide, norepinephrine transporter gene and ¹³¹I-metaiodobenzylguanidine, and the human sodium iodide symporter (hNIS) gene and radioiodines or ^{99m}Tc-pertichnetate. Of these, it has been suggested that the NIS gene system is the most straightforward.¹²⁵

NIS is a transmembrane glycoprotein consisting of 13 transmembrane segments, an extracellular N-terminus domain and an intracellular C-terminus.¹³⁰ It is found naturally in a number of human tissues: mainly the thyroid but in others too, including the stomach, salivary glands, mammary glands and extraocular muscles.¹³¹ It is a member of the sodium/solute symporter family but can transport many anions in addition to iodide.¹³² Its ability to transport radioactive iodide and other radiolabelled anions such as ^{99m}TcO₄⁻ and ⁷⁶Br⁻ has suggested its potential for use in diagnostics and as a reporter gene for GDEPT.^{132,133} As it is a human gene it should not cause an adverse immune response. Its substrates are easily available, as is information on their metabolisms. Unlike D2R and HSVtk, labelling of the substrates of NIS is stable. However, the use of the NIS gene system is limited in specific tissues by its endogenous expression which would reduce sensitivity and specificity (although this should not affect its potential use in the prostate). It is also restricted by the swift loss of iodide from hNIS-expressing cells.^{125,134}

Recently a phase I study has examined the feasibility of using a therapeutic adenovirus, monitored non-invasively through the utilisation of the sodium iodide transporter.¹³⁵ Replication-competent Ad5-yCD/*mutTK*_{SR39}*rep*-hNIS was injected into the prostate of men with clinically localised prostate cancer. This was followed by administration of the radioactive tracer sodium pertechnetate (Na^{99m}TcO₄), allowing imaging of NIS gene expression using single photon emission-computed tomography (SPECT). Using this method, Barton et al. were able to calculate the volume, total amount and persistence of gene expression in the prostate. This gene expression was not detected in any tissue outside the prostate and none of the adverse events (98% Grade 1 or 2) was deemed to be caused by the nuclear imaging.

Barton et al. also highlighted the advantages of this particular reporter gene system. Since the hNIS gene is under the control of cytomegalovirus promoter, which is ubiquitous, NIS expression is not restricted to any tissue in particular. This is in contrast to reporter genes that are under the control of prostate specific promoters and therefore could not give information about the spread of the virus outside the prostate. In the case of this particular adenovirus, Barton et al. noted that a variety of imaging techniques could be utilised as CD and HSVtk can also be imaged noninvasively as previously discussed. Therefore, the most suitable reporter system for the tissue in question could be chosen.

Conclusion

Gene-directed enzyme prodrug therapy (GDEPT) has the potential to improve prostate cancer treatment outcomes in patients receiving radiation therapy. The results of the initial clinical trials discussed here are promising, demonstrating the safety and in some cases, the efficacy of GDEPT strategies for the treatment of various stages of prostate cancer. Further trials, including phase III studies, are needed to firmly establish the potential benefit of these treatments. A number of the problems limiting the progress of GDEPT have been discussed. Of note, techniques to allow non-invasive imaging of transgene expression *in vivo* are being developed and are necessary to enable the initial assessment of the success of the treatment. Improving vector delivery is also important for the advancement of GDEPT.

It is clear that an important consideration in the development of a gene therapy strategy is the decision on what patient cohort should be targeted. In this review we have discussed the gene therapy clinical trials involving both localised and metastatic disease. However improvements in systemic treatments for metastatic cancer are only necessary because primary treatment fails in a high proportion of cases.⁷⁹ Therefore, it is logical to seek improvements

in treatment of localised cancer and restrict its spread. The clinical trials discussed here suggest that treatment of localised disease with radiotherapy could be more effective if combined with GDEPT. We see the future of GDEPT as being part of a multi-modal treatment strategy in which it works to improve the efficacy of the current methods of prostate cancer treatment.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by the Irish Health Research Board and the Irish Cancer Society.

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Gene Expression Analysis in Prostate Cancer: The Importance of the Endogenous Control

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BACKGROUND. Aberrant gene expression is a hallmark of cancer. Quantitative reverse-transcription PCR (qRT-PCR) is the gold-standard for quantifying gene expression, and commonly employs a house-keeping gene (HKG) as an endogenous control to normalize results; the choice of which is critical for accurate data interpretation. Many factors, including sample type, pathological state, and oxygen levels influence gene expression including putative HKGs. The aim of this study was to determine the suitability of commonly used HKGs for qRT-PCR in prostate cancer.

METHODS. Prostate cancer (LNCaP, 22Rv1, PC3, and DU145) and normal (PWR1E and RWPE1) cell lines were cultured in air and hypoxia. The performance of 16 HKGs was assessed using Normfinder and coefficient of variation. In silico promoter analysis was performed to identify putative hypoxia response elements (HREs). The impact of the endogenous control on expression levels of *HIF1A* and *GSTP1* was investigated by qRT-PCR in cell lines and tissue specimens respectively.

RESULTS. Hypoxia altered expression of several HKGs: *IPO8*, *B2M*, and *PGK1*. The most stably expressed HKGs were *ACTB*, *PPIA*, and *UBC*. Both *UBC* and *ACTB* showed constitutive expression of *HIF1A* in air and hypoxia, while *PGK1* falsely implied a sixfold hypoxia-induced down-regulation. In prostate tumors, *UBC* and *PGK1* both revealed down-regulation of *GSTP1* relative to matched benign, whereas *ACTB* showed variability.

CONCLUSIONS. This study demonstrates that no universal endogenous control exists for gene expression studies, even within one disease type. It highlights the importance of validating expression of intended HKGs between different sample types and environmental exposures. *Prostate* © 2012 Wiley Periodicals, Inc.

KEY WORDS: qRT-PCR; house-keeping gene; prostate cancer; endogenous control

INTRODUCTION

Quantitative or real-time reverse transcription PCR (qRT-PCR) is a commonly used laboratory technique in cancer research, enabling the sensitive quantification of gene expression levels [1]. However, it holds a number of potential pitfalls, one of which is normalization of gene expression results. This is of paramount importance, as there are multiple factors related to the processes of RNA extraction, reverse transcription, and PCR that can introduce variation

Additional supporting information may be found in the online version of this article.

Grant sponsor: Irish Health Research Board; Grant sponsor: Irish Cancer Society; Grant sponsor: Prostate Cancer Foundation.

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Received 7 June 2012; Accepted 2 August 2012

DOI 10.1002/pros.22578

Published online in Wiley Online Library
(wileyonlinelibrary.com).

between samples. These include the source and amount of starting material, RNA integrity, the method of RNA extraction and whether a DNase step is performed [2,3]. Several different normalization methods exist. Using a similar sample size of tissue or number of cells from in vitro experiments is helpful but not sufficient [4]. Standardizing the quantity of total RNA between samples is important to ensure that equal amounts of RNA are used for reverse transcription (the quality of the RNA can also be assessed at this stage) but does not account for potential variability in the downstream PCR step, such as differences in reverse transcriptase efficiency between individual reactions.

The most commonly used method of normalization involves using an endogenous control or “house-keeping” gene (HKG), whose expression does not vary between samples and is independent of the pathological state or exposure to environmental stresses or changes in growth conditions. This technique controls for variation in all stages of the procedure up to and including the qRT-PCR measurement. Traditionally this has involved the use of a number of genes such as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) or β -actin (*ACTB*) and the assumption of their constant expression. Huggett et al. [4] suggest that this stems from the traditional use of these genes as controls for experiments like Northern blots. However, it is well established that the expression of these genes can vary and may be affected by tissue type and different treatment conditions [4–6]. So, while certain classical HKGs are often employed to normalize qRT-PCR experiments, it is imperative that their steady-state level of expression is carefully validated under each experimental condition being tested (i.e., disease phenotype, drug exposure, etc.). If this step is not carried out, it can lead to selection of a gene whose fluctuation in expression will mask small changes in the expression of the target genes being quantified. If the control gene is even moderately affected by the different experimental conditions being studied, it can result in erroneous findings, not necessarily obvious to the researcher.

Aberrant gene-expression is a prominent feature of prostate cancer, with overexpression of genes such as *AMACR*, *PCA3*, and loss or down-regulation of other genes including *GSTP1*. Whole-genome transcriptome studies have revealed stark differences in expression profiles between tumor and benign prostate and between different stages of prostate carcinogenesis [7]. Validation of these findings and moreover, functional studies on individual genes, largely utilize qRT-PCR. There are a number of confounding factors in relation to prostate cancer that can affect the suitability of potential HKGs, one of which is the sample type used in

these studies. For example, due to the intrinsic difficulty in identifying tumor in fresh prostate, most gene expression analysis is done on formalin fixed paraffin embedded (FFPE) prostate cancers. However, it is widely accepted that this process can have a profound effect on RNA integrity. Another important factor is the tumor microenvironment, of which the effects of hypoxia are particularly well characterized. Hypoxia (abnormally low levels of oxygen) induces numerous stress related signaling pathways in the cell. This is mediated by an inducible transcription factor, hypoxia inducible factor 1 (HIF-1), which exists as a heterodimer of HIF-1 α and HIF-1 β [8]. Both proteins are constitutively expressed. HIF-1 β is stable, regardless of oxygen concentration, but the presence of HIF-1 α is determined at the protein level by the amount of oxygen [9]. Under normoxic but not hypoxic conditions, HIF-1 α is degraded by the proteasome.

It is estimated that up to 5% of all genes are activated by HIF-1 and these genes are involved in a diverse range of processes including cell survival, oxygen transport, apoptosis, and angiogenesis [9,10]. Direct activation of a proportion of these genes occurs through HIF-1 binding to enhancers known as hypoxia response elements (HREs) contained in their 5' regulatory regions and promoters. HREs contain the conserved core sequence 5'-(A/G)CGT(G/C)(G/C)-3'. Activation of the HREs initiates the cellular response to hypoxia, leading to increased oxygen delivery and aiding in survival [11,12].

HIF-1 overexpression is a prominent feature of prostate cancer, as well as many other cancer types and is significantly associated with cancer progression [11,12]. The rapid growth of tumors results in a disorganized vascular network, which cannot keep pace with the oxygen demand. Blood flow may be impaired by malformations of the blood vessels meaning oxygen cannot reach some parts of the tumor, causing them to be hypoxic [13–15]. Hypoxia has been associated with tumor aggressiveness and metastasis due to the upregulation of a host of genes involved in angiogenesis and glycolysis. Consequently, hypoxia is associated with poor prognosis in several cancer types, including prostate [11,15]. It has also been associated with resistance to both chemotherapy and radiation therapy [12].

In this study, we tested the performance of 16 commercially available endogenous control genes in two prostate cancer cell lines (LNCaP and 22Rv1) cultured under both hypoxic and normoxic conditions. Statistical analysis revealed wide variation in expression for many of the endogenous controls. In silico analysis of the regulatory regions of these genes revealed putative HREs in several of them. Four genes were selected for further analysis in a broader panel of prostate

cell lines, DU145, PC3, PWR1E, and RWPE1. *UBC* was the most consistent of the potential control genes, performing well in all cell lines tested. *ACTB* performed reasonably well in the cell lines but it was not suitable as a control for prostate tissue specimens, with a high standard deviation between expression results for the gene *GSTP1*.

MATERIALS AND METHODS

Cell Culture

The cell lines 22Rv1, LNCaP, DU145, PC3, PWR1E, and RWPE1 were obtained from the American Type Culture Collection. 22Rv1 and LNCaP cells were grown in RPMI 1640 GlutaMax (Gibco, USA), 1% penicillin/streptomycin and 10% charcoal dextran stripped fetal bovine serum (FBS, Gibco). The remaining cell lines were grown in RPMI medium with 10% FBS and 1% penicillin/streptomycin. The cells were cultured for 24 hr in a humidified incubator (approx. 21% oxygen) or in a hypoxia chamber (BioTrace, UK) in which levels of oxygen were 0.5% with 5% carbon dioxide.

RNA Extraction and cDNA Synthesis

TRI reagent (Sigma) was used to isolate total RNA, which was subsequently DNase treated (Ambion). A Nanodrop 1000 spectrophotometer was used to measure the concentration and purity of the RNA in each sample. cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) on 250–1,000 ng of RNA, following the manufacturer's protocol.

Tissue Samples

Prostate tissue specimens (tumor and histologically benign) from 11 men undergoing radical prostatectomy for primary prostate cancer were obtained through the histopathology archives dating from 2000 at St. James's Hospital. Suitable formalin fixed paraffin embedded (FFPE) blocks of each case were selected and corresponding Hematoxylin and Eosin (H&E) stained sections were reviewed and annotated by a pathologist. A series of 5 μ m sections were cut from the FFPE blocks. The first and last sections were H&E stained and compared with the pathologically evaluated slides. Intervening sections were deparaffinized; tissue was scraped from within the marked target areas and total RNA was extracted using a Recover-All™ Total Nucleic Acid Isolation Kit (Ambion Inc., Austin, TX) and quantified using a Nanodrop-1000 Spectrophotometer (Labtech International, UK).

Human Endogenous Control Plate and qRT-PCR

Human endogenous control array cards (Part number: 4367563, Applied Biosystems) were prepared and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's guidelines. 2 ng/ μ l (200 ng total) of cDNA was used for each sample.

The PCR reaction master mix contained 0.5 μ l 20 $\times$ TaqMan Gene Expression Assay, 5 μ l TaqMan Gene Expression Master Mix and 3.5 μ l RNase free water. It was combined with 1 μ l of each cDNA samples in a 96-well plate (MicroAmp Optical reaction plate, Applied Biosystems). The $\Delta\Delta C_t$ method was used to calculate the relative quantity of gene expression. The 7500 Software v2.01 and RQ Manager v1.2 were used to analyze results.

In Silico Prediction of HREs

A database containing information on transcription factor binding sites (TFBS) in the proximal promoter regions of ~20,000 genes has previously been published [16]. This database contains the TFBS information for approximately 1,236 known and predicted TFBS conserved across human, mouse, rat and dog in the proximal promoter regions of these genes. In this case, the proximal promoter is defined as 2 kb up and downstream of the Refseq annotated transcription start site [17]. Included among these TFBS are the well characterized TFBS for HIF-1 α and HIF-1 β [18]. The likelihood that the control genes contained HREs was assessed at four different position specific scoring matrix (PSSM) thresholds, 0.7, 0.75, 0.8, 0.85, each representing different degrees of sensitivity and selectivity. The higher the PSSM threshold the greater the similarity of the HRE to the previously experimentally characterized HREs. Promoters for 18S rRNA and IPO8 were not in the database and could not be included in this analysis.

Data Analysis

The freely available statistical programme Normfinder [2] was used to rate the suitability of genes as endogenous controls. Normfinder allows for assessment of both inter- and intragroup variability and ranks the genes accordingly to give a measure of stability. With this method, the cycle threshold (C_t) variation within the air/hypoxia sample groups was assessed for each gene as well as the variation between air and hypoxia groups. The coefficient of variation ($C_tCV\%$) compares the variation in one sample set to another and was used to test the performance of smaller sets of HKGs, when use of Normfinder is not appropriate. The $C_tCV\%$ (standard

deviation/mean) is expressed as a percentage and gives a measure of the spread of the C_t values from the mean.

RESULTS

Optimal Endogenous Control Genes in Air and Hypoxia

The performance of 16 commercial endogenous control genes for qRT-PCR was tested in two prostate cancer cell lines, 22Rv1 and LNCaP (Table I). The cell lines were grown in air and hypoxia (0.5% oxygen) for 24 hr before RNA extraction. The raw C_t values for the two treatment groups are given in Table II. The results for cells grown under aerobic conditions were analyzed using Normfinder and the genes were ranked based on their variability (Table III). The C_t data were further analyzed to test the variability of the endogenous control genes under both aerobic and hypoxic conditions (Table III). A significant effect of hypoxia on gene expression was demonstrated by a shift in the ranking of endogenous gene stability when the hypoxic and aerobic samples were analyzed together compared to the aerobic samples alone. *PGK1* was one of the top ranked control genes under aerobic conditions, but had the highest variability and was the least suitable control gene for both cell lines when hypoxic and aerobic samples were analyzed together.

There was some disparity between the two cell lines, 22Rv1 and LNCaP, most notably for the genes

POLR2A and *YWHAZ*. Analysis using Normfinder showed that, of the 16 HKGs analyzed, *POLR2A* performed the best in 22Rv1, with the lowest variability at 0.2026. For LNCaP, however, it was ranked 15th, with a variability of 0.8485. Conversely, *YWHAZ* was the best control gene in LNCaP cells (variability = 0.2238) but was ranked 12th for 22Rv1 (variability = 0.7686).

The three endogenous control genes that performed best under air and hypoxia in both cell lines (*ACTB*, *PPIA*, and *UBC*), were selected for further testing, along with the worst performer (*PGK1*; Fig. 1) in another prostate cancer cell line, DU145. For this analysis on 3 HKGs, Normfinder was unsuitable so the coefficient of variation ($C_tCV\%$) between the two treatment groups (air and hypoxia) was calculated for the average C_t values of the 3 biological replicates within each group. Of the four genes, *PPIA* performed the best, with a $C_tCV\%$ of 0.53, whilst *PGK1* remained the poorest ($C_tCV\%$ = 7.44; Table IV).

Performance of Selected Endogenous Control Genes Following Longer Hypoxia Exposure

In the initial experiments, cells were cultured under aerobic or hypoxic conditions for 24 hr. To assess whether length of hypoxia exposure would affect the performance of the selected endogenous control genes, further analysis was carried out on 22Rv1, LNCaP, and DU145 cells grown in air or hypoxia for 48 hr before RNA extraction and assessed by $C_tCV\%$. Longer hypoxic exposure did not alter the ranking of

TABLE I. Genes Tested for Suitability as Endogenous Control Genes for qRT-PCR in Prostate Cancer

Symbol	Gene name	Function
18S rRNA	18s ribosomal RNA	Ribosome subunit
ACTB	β-actin	Cytoskeletal structural protein
B2M	β-2-microglobulin	β-chain of the major histocompatibility complex class I molecules
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis
GUSB	β-glucuronidase	Lysosome exoglycosidase
HMBS	Hydroxymethylbilane synthase	Heme biosynthesis
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Purine generation through the purine salvage pathway
IPO8	Importin 8	Nuclear protein import
PGK1	Phosphoglycerate kinase 1	Glycolysis enzyme
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	Synthesis of messenger RNA
PPIA	Cyclophilin A	Serine-threonine phosphatase inhibitor
RPLPO	Ribosomal protein, large, P0	Ribosome subunit
TBP	TATA box binding protein	Initiation of transcription by RNA polymerase II
TFRC	Transferrin receptor (p90, CD71)	Cellular iron uptake
UBC	Ubiquitin C	Ubiquitination
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Signal transduction

TABLE II. Mean C_t Values for Endogenous Control Genes

	22Rv1		LNCaP	
	Air C _t (SD)	Hypoxia C _t (SD)	Air C _t (SC)	Hypoxia C _t (SD)
18S	9.65 (0.53)	10.31 (2.42)	13.1 (2.98)	10.07 (0.3)
ACTB	19.53 (0.05)	20.82 (1.94)	23.44 (1.27)	22.39 (0.41)
B2M	24.05 (0.13)	25.59 (2.92)	26.33 (1.62)	25.61 (0.9)
GAPDH	19.56 (0.19)	19.18 (2.07)	23.7 (2.42)	20.53 (0.99)
GUSB	24.42 (0.12)	25.55 (1.48)	26.27 (0.59)	25.86 (0.6)
HMBS	26.03 (0.12)	27.17 (1.62)	28.48 (1.24)	27.81 (0.29)
HPRT1	24.67 (0.02)	26.44 (3.39)	30.43 (2.35)	29.19 (0.68)
IPO8	25.52 (0.04)	27.94 (3.99)	27.8 (0.68)	27.24 (0.38)
PGK1	23.78 (0.08)	21.78 (1.05)	25.55 (1.39)	21.68 (0.91)
POLR2A	24.45 (0.11)	25.34 (1.92)	26.3 (0.14)	26.53 (0.62)
PPIA	20.22 (0.09)	21.06 (1.38)	22.93 (1.55)	22.24 (0.4)
RPLPO	20.44 (0.14)	20.58 (1.03)	23.75 (2.79)	21.46 (0.59)
TBP	27.66 (0.03)	29.48 (3.03)	32.66 (3.72)	30.62 (0.75)
TFRC	23.99 (0.08)	25.93 (3.49)	26.85 (1.25)	25.81 (0.65)
UBC	22.54 (0.19)	23.13 (1.51)	26.27 (2.07)	24.56 (0.42)
YWHAZ	24.01 (0.16)	25.65 (3.56)	27.53 (1.4)	26.29 (0.72)

SD, standard deviation. Values represent the mean of 3 biological replicates.

the four endogenous controls by variability for any of the cell lines (Fig. 2A).

The performance of the three selected endogenous control genes was tested in three additional cell lines: PC3, a metastatic prostate cancer cell line and the normal prostate lines PWR-1E and RWPE-1. The cells were grown in air or hypoxia for 48 hr prior to RNA extraction. The C_tCV% values for PC3 were not

consistent with the previous results for 22Rv1, LNCaP, and DU145, with *PGK1* appearing the most suitable endogenous control of the four genes tested (C_tCV% = 0.34). The best performing genes for PWR-1E and RWPE-1 were *UBC* (C_tCV% = 1.27) and *PPIA* (C_tCV% = 1.15), respectively (Fig. 2B).

A large degree of variation between cell lines was seen in the performance of the potential endogenous

TABLE III. Endogenous Control Genes Ranked by Their Stability in Hypoxic and Aerobic Samples Using NORMFINDER

22Rv1					LNCaP				
Rank	Air		Air and Hypoxia		Rank	Air		Air and Hypoxia	
	Gene	Variability	Gene	Variability		Gene	Variability	Gene	Variability
1	ACTB	0.0136	POLR2A	0.2026	1	B2M	0.0366	YWHAZ	0.2238
2	TBP	0.0136	ACTB	0.2735	2	PPIA	0.0366	UBC	0.2725
3	PGK1	0.0335	HMBS	0.3812	3	YWHAZ	0.1839	TFRC	0.2924
4	HPRT1	0.0492	18S	0.4248	4	PGK1	0.2055	ACTB	0.3011
5	IPO8	0.0689	GUSB	0.4255	5	UBC	0.3019	HPRT1	0.3843
6	HMBS	0.0907	PPIA	0.4438	6	ACTB	0.3729	PPIA	0.4654
7	POLR2A	0.1036	UBC	0.5213	7	TFRC	0.3992	RPLPO	0.5158
8	GUSB	0.1193	B2M	0.5574	8	HMBS	0.4217	B2M	0.5238
9	TFRC	0.1202	TBP	0.6488	9	HPRT1	0.647	GUSB	0.5489
10	PPIA	0.1285	HPRT1	0.7182	10	GAPDH	0.7268	IPO8	0.6062
11	RPLPO	0.1418	GAPDH	0.7631	11	IPO8	1.0552	HMBS	0.6203
12	B2M	0.1601	YWHAZ	0.7686	12	RPLPO	1.1392	TBP	0.7034
13	UBC	0.1618	RPLPO	0.7973	13	GUSB	1.1622	GAPDH	0.8252
14	GAPDH	0.1675	TFRC	0.8274	14	18S	1.3449	18S	0.8473
15	YWHAZ	0.2074	IPO8	1.0709	15	POLR2A	1.9466	POLR2A	0.8485
16	18S	0.5269	PGK1	1.5052	16	TBP	2.1437	PGK1	0.9536

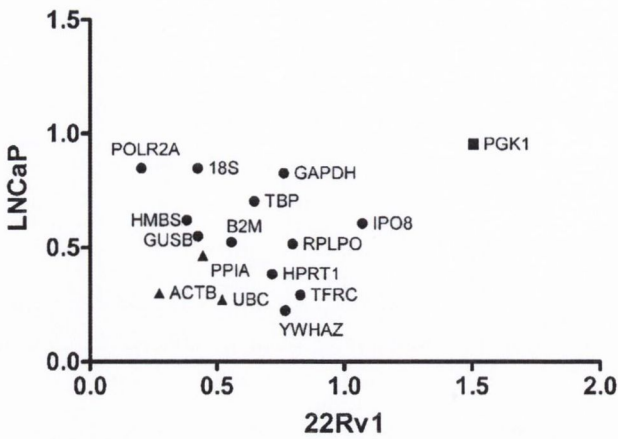


Fig. 1. Variability of 16 endogenous control genes in prostate cancer cell lines 22Rv1 and LNCaP cultured under aerobic or hypoxic conditions. The three best performing genes (*ACTB*, *UBC*, *PPIA*, triangles), and the worst performing gene (*PGK1*, square) are indicated and were selected for further analysis.

control genes. The most consistent genes were *UBC*, with the lowest C_t CV% across all six cell lines, range: 1.09 (22RV1)—3.52 (PC3) and *ACTB*, C_t CV range: 0.33 (22RV1)—7.82 (PC3).

The Choice of Endogenous Control Can Result in Variation in Gene Expression Results

To demonstrate the importance of the endogenous control in gene expression analysis, we selected two of the best performing genes (*ACTB* and *UBC*) along with *PGK1*, whose expression we found to be altered by hypoxia in certain prostate cancer cell lines, and used each of them to normalize expression of the hypoxia-induced transcription factor *HIF-1A* in 22Rv1 cells cultured in air and hypoxia (Fig. 3). Both *ACTB* and *UBC* showed similar levels of *HIF-1A* expression in aerobic and hypoxic cells, confirming constitutive expression of the gene. However, when *PGK1* was used to normalize results, an apparent sixfold decrease in *HIF-1A* expression occurred under hypoxia ($P < 0.001$). This erroneous interpretation is directly due to the upregulation of *PGK1* under hypoxic conditions, leading to earlier amplification and hence

lower C_t values compared to aerobic conditions (Table II). Consequently, *HIF-1A* gene expression is falsely found to be down-regulated under hypoxic conditions.

In Silico Analysis of Hypoxia Response Elements

We further dissected the potential involvement of hypoxia and more specifically HIF1A in transcriptionally regulating HKG expression by performing an in silico analysis of the proximal promoters of the 16 endogenous control genes to identify putative HREs (Supplementary Table I). At the highest position specific scoring matrix (PSSM) threshold of 0.85, *PGK1*, *GAPDH*, and *HMBS* were positive for a *HIF-1A* binding site in their proximal promoter. Both *PGK1* and *GAPDH* performed poorly as endogenous control genes for 22Rv1 and LNCaP. *HMBS* had high levels of variability in LNCaP but performed well for 22Rv1. The presence of HREs in their promoters suggests that these genes may be regulated in response to hypoxia, calling into question their use as endogenous controls under hypoxic conditions. However the results indicate that this in silico analysis alone is not sufficient to predict the variability of the potential endogenous control genes between aerobic and hypoxic samples.

Performance of Selected Endogenous Control Genes in Tumor and Normal Tissue

Finally, we tested the performance of the four prostate HKGs (*UBC*, *PPIA*, *ACTB*, and *PGK1*) in 11 matched tumor and benign FFPE prostate tissue specimens, by measuring expression of intracellular detoxification enzyme and bone-fide marker of prostate cancer, *GSTP1*. It has been widely established that *GSTP1* is down-regulated in more than 90% of prostate tumors as a result of hypermethylation [19]. Using *PPIA*, *UBC*, or *PGK1* as endogenous controls showed *GSTP1* levels were 2.5- to 3.9-fold lower in tumor samples relative to matched normal tissue (Fig. 4). However, when normalized to *ACTB*, *GSTP1* was found to be up-regulated in 3/11 tumors and also displayed a large standard deviation, which indicates its unsuitability as a HKG in FFPE tissues.

TABLE IV. Assessment of Performance of HKGs Using Coefficient of Variation Analysis in DU145 Cells					
	Average C_t air	Average C_t hypoxia	Average C_t air and hypoxia	SD	C_t CV%
<i>PPIA</i>	19.45	19.59	19.52	0.1	0.53
<i>UBC</i>	21.54	22.24	21.89	0.49	2.25
<i>ACTB</i>	18.88	20.11	19.5	0.86	4.43
<i>PGK1</i>	21.67	19.51	20.59	1.53	7.44

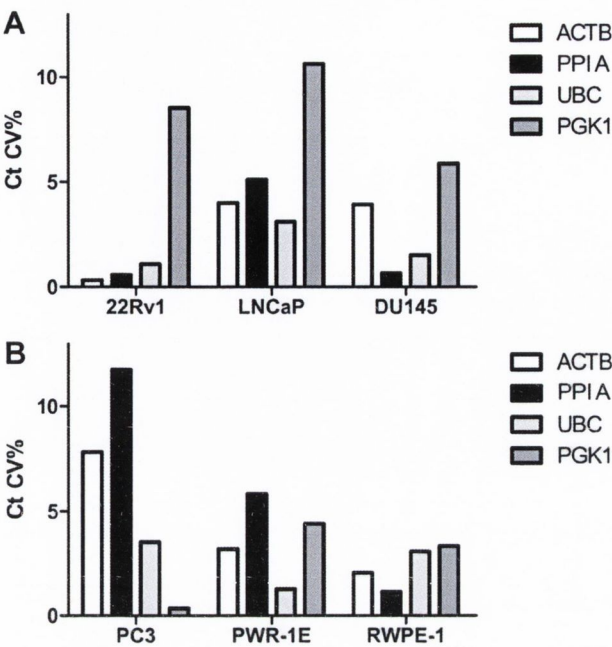


Fig. 2. **A:** Coefficient of variation of C_t values between aerobic and hypoxic treatment groups for four candidate endogenous control genes in three prostate cancer cell lines following longer hypoxic exposure (48 hr). **B:** Three additional prostate cell lines were tested after growth under aerobic or hypoxic conditions for 48 hr. C_t CV%, coefficient of variation.

DISCUSSION

This research highlights the importance of thorough investigation to find a suitable endogenous control gene before proceeding with qRT-PCR experiments. A growing number of research groups, including Bustin and colleagues [20], have demonstrated this and have drawn attention to the significance of the choice of endogenous control gene. Unfortunately, it is a consideration that is often overlooked.

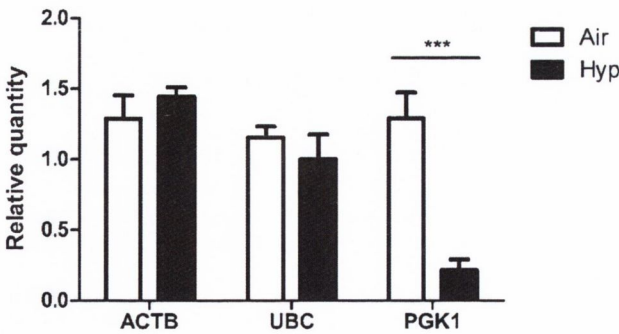


Fig. 3. Relative quantification of *HIF-1A* mRNA in 22Rv1 using 3 different endogenous controls *ACTB*, *UBC*, and *PGK1*. Values represent mean of three replicates $\pm$ standard error of the mean. *** $P < 0.001$.

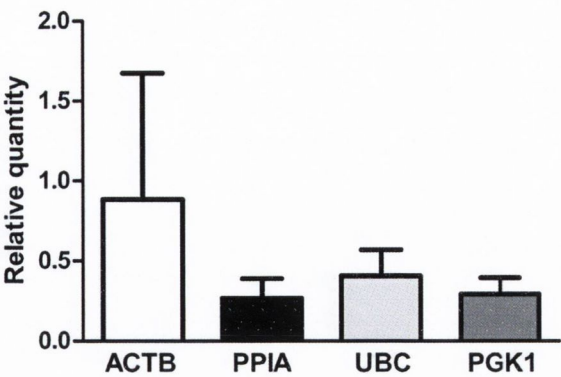


Fig. 4. Average *GSTP1* mRNA expression levels in tumor samples relative to matched normal tissue using four different endogenous control genes. Values represent the mean of 11 replicates $\pm$ standard deviation.

What has become clear is that no universally suitable endogenous control gene exists; a gene shown to be suitable in one tissue type or cell line will not necessarily work for another. This research found some consensus between cell lines but some obvious disparity too. As well as cell line to cell line variation, exposure to different treatment conditions can influence expression of potential endogenous control genes. We addressed the possible effect hypoxia could have on the expression of 16 endogenous control genes, considering that hypoxia has been shown to influence expression of a wide-ranging number of genes. This study clearly demonstrates that it also modulates transcription of housekeeping genes, in particular *PGK1*. In silico analysis of the 16 endogenous control genes was carried out to assess the likelihood that they contain HREs in their promoters. Using this technique, *PGK1* and *GAPDH* were both shown to have predicted HREs, which supports previous findings [21,22] and explains the high variability for these genes between aerobic and hypoxic samples. However, *HMBS* and *POLR2A* also showed strong consensus with the HRE sequence and yet performed as two of the best endogenous control genes for 22Rv1 (but not for LNCaP). These results point towards the complexity of hypoxia-mediated gene regulation and the heterogeneity of its effect in different cell lines.

The considerable effect that an endogenous control gene can have on the final gene expression results was demonstrated by using several different HKGs to normalize *HIF-1A* gene expression in aerobic and hypoxic 22Rv1 cell samples. It is known that *HIF-1 α* expression is controlled at the protein level and that transcription does not vary between normoxia and hypoxia [9]. When either *ACTB* or *UBC* were used as the reference gene, *HIF-1A* gene expression was not

significantly altered by hypoxia. However when *PGK1* was used to normalize the data, *HIF-1A* was incorrectly found to be substantially downregulated by hypoxia, a result, which is in fact due to the upregulation of *PGK1* under hypoxic conditions.

Gene expression can be modulated, in part, by the local tissue microenvironment leading to differences in expression levels between histologically benign tissue adjacent and tumor tissue and differences compared to tissue from men without prostate cancer. Changes in the growth and metabolism of tumor cells can also mean differences in gene expression compared to normal tissue. The careful choice of endogenous control gene is just as crucial for qRT-PCR analysis of gene expression in tissue samples. It is also important to re-assess the suitability of endogenous control genes when moving from cell lines to tissue samples or vice versa. In this study *ACTB* was one of the best performing control genes for a number of prostate cancer cell lines. However, in archived patient tissue, it did not perform as well for some samples, with relatively high C_t values. The four candidate endogenous control genes, *ACTB*, *PPIA*, *UBC*, and *PGK1* were used to normalize results for expression of *GSTP1* in the tissue samples. *GSTP1* has been previously demonstrated to be downregulated in prostate tumor samples compared to normal tissue [19]. This finding was replicated here in all 11 tissue samples compared to the matched normal tissue when *PPIA*, *UBC*, or *PGK1* were used as the endogenous control. However, when using *ACTB*, *GSTP1* was incorrectly found to be upregulated for three of the samples. These findings support those of Ohl et al. [23] who found a significant difference in *ACTB* expression between matched malignant and benign prostate tissue. Erickson et al. [24] suggested *ACTB* may be a good endogenous control gene for prostate cell line samples but not for tissue, which may be a result of differences in growth conditions between the two. In vitro growth conditions lead to steady high expression of *ACTB* in cell lines, whereas this high level of expression may not be seen in tissue samples and this would affect the accuracy of results.

Mounting evidence highlights the importance of careful selection of the endogenous control genes in qRT-PCR experiments. However, such considerations are often overlooked. Failure to include this step means one cannot be confident in the ultimate gene expression findings, which may include erroneous and misleading results. This study shows that the expression of some common endogenous control genes varies between aerobic and hypoxic samples, making them unsuitable for normalization of qRT-PCR results. Hypoxia is a common characteristic of solid tumors and a greater understanding of its effects

on gene expression will improve our understanding of tumor biology. However, any study involving qRT-PCR must use endogenous control genes that have been carefully validated.

ACKNOWLEDGMENTS

We gratefully acknowledge funding from the Irish Health Research Board, the Irish Cancer Society, and the Prostate Cancer Foundation.

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