A protease resistant insulin like growth factor binding protein 4 as a treatment for prostate cancer

Gemma Kirwan
Royal College of Surgeons in Ireland

Citation
Creative Commons Licence:

This work is licensed under a Creative Commons Attribution-Noncommercial-Share Alike 3.0 License.

This thesis is available at e-publications@RCSI: http://epubs.rcsi.ie/phdtheses/58
A protease resistant Insulin-like growth factor binding protein 4 as a treatment for prostate cancer

A thesis submitted for the degree of Doctor of Philosophy
By
Gemma Kirwan, B.A. (Mod)

Presented to the Royal College of Surgeons in Ireland

(National University of Ireland)

Under the supervision of Dr. Judith H. Harmey

Department of Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland,
123 St. Stephen's Green, Dublin 2.
May 2012

This work was funded by the HRB in Ireland under Grant No. PHD/2007/11.
Table of Contents

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>i</td>
</tr>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>iv</td>
</tr>
<tr>
<td>Units</td>
<td>x</td>
</tr>
<tr>
<td>Abstract</td>
<td>xi</td>
</tr>
</tbody>
</table>

Chapter 1: General Introduction

1.0 Introduction 2

1.1 Cancer 2

1.2 Development of cancer 2

1.3 Prostate cancer 3

1.3.1 Risk factors of prostate cancer 3

1.3.1.1 Age 3

1.3.1.2 Family history 4

1.3.1.3 Race/ethnicity 4

1.3.1.4 Diet 4

1.3.2 Treatment of prostate cancer 5

1.3.2.1 Active surveillance 5

1.3.2.2 Surgery 5

1.3.2.3 Radiation therapy 6

1.3.2.3.1 External beam therapy 6

1.3.2.3.2 Brachytherapy 6

1.3.2.4 Chemotherapy 6

1.3.2.5 Hormone therapy 7

1.4 Androgen Independent prostate cancer 7

1.4.1 Hypersensitive pathway 8

1.4.2 Promiscuous pathway 8

1.4.3 Outlaw pathway 9

1.4.4 Bypass pathway 10

1.5 Metastasis 10

1.5.1 Bone metastasis 11
1.6 Insulin-like growth factor (IGF) pathway
   1.6.1 Insulin-like growth factor binding proteins (IGFBPs) 14
   1.6.2 IGF pathway and prostate cancer 15
   1.6.3 Targeting the IGF pathway in cancer 17
      1.6.3.1 Therapies targeting the IGF ligand 18
      1.6.3.2 Anti-sense oligonucleotides and RNAi 22
      1.6.3.3 Monoclonal antibodies to inhibit IGF I and the IGF IR 23
      1.6.3.4 Tyrosine kinase inhibitors to block IGF IR activation 26
1.7 Animal models of prostate cancer 28
   1.7.1 Non-invasive imaging of animal models of cancer 30
1.8 Overall aims 34
   1.8.1 Specific aims 34

Chapter 2: Materials and Methods 35
2.1 Reagents 36
2.2 Cell culture 36
   2.2.1 Cell lines 36
   2.2.2 Subculture of PC-3M-luc2, HEK 293 and HEK293 T cells 36
   2.2.3 Preparation of frozen cell stocks 37
   2.2.4 Revival of frozen cells 37
   2.2.5 Cell counting assay 37
   2.2.6 Single cell cloning by serial dilution 38
   2.2.7 Mycoplasma testing 38
      2.2.7.1 MyoAlert assay 38
      2.2.7.2 Mycoplasma PCR 40
2.3 Tissue microarray (TMA) construction 40
   2.3.1 Immunohistochemical staining of TMAs 41
   2.3.1.1 IGF IRβ staining of TMAs 41
   2.3.1.2 Haematoxylin and Eosin staining of TMAs 41
2.4 CD31+ staining of human endothelial cells 41
2.5 RNA and DNA analysis 42
   2.5.1 Isolation of RNA 42
   2.5.2 RNA/DNA quantification 43
   2.5.3 cDNA synthesis 43
   2.5.4 Primer design 43
   2.5.5 Polymerase chain reaction (PCR) 44
   2.5.6 DNA electrophoresis 45
2.6 Molecular cloning

2.6.1 DNA recovery from gel
2.6.2 DNA digestion with restriction enzymes
2.6.3 Ethanol precipitation of DNA
2.6.4 DNA ligation
2.6.5 Bacterial transformation
  2.6.5.1 Media/solutions
  2.6.5.2 Transformation of bacterial cells
2.6.6 Colony PCR
2.6.7 Plasmid purification from bacterial cultures
2.6.8 Sequencing of cloned inserts

2.7 Mammalian cell transfection and transduction

2.7.1 Transfection of HEK 293 T cells with pLVX pTet-On Advanced and pLVX Tight Puro dBp4
2.7.2 Transduction of PC-3M-luc2 cells with pLVX pTet-On Advanced and pLVX Tight Puro dBp4

2.8 Luciferase assay

2.8 Protein analysis

2.9.1 Collection and storage of cell lysates and conditioned medium
2.9.2 Protein quantification using Bio-Rad DC protein assay
2.9.3 Immunoprecipitations
2.9.4 Protein purification
  2.9.4.1 Nickel agarose purification of recombinant protease resistant IGFBP 4 (dBp4)
  2.9.4.2 Fast protein liquid chromatography
2.9.5 PAPP-A and PSA digest of protease resistant IGFBP 4
2.9.6 Western blotting of proteins
2.9.7 Silver staining of SDS-PAGE gel
2.9.8 Western blot transfer
2.9.9 Ponceau S staining
2.9.10 Antibody detection for Western blotting
2.9.11 IGF binding ELISA
2.9.12 VEGF<sub>165</sub> ELISA
2.9.13 IGF I ELISA
2.9.14 PAPP-A ELISA
2.10 Measurement of cell proliferation
   2.10.1 BrdU proliferation assay
   2.10.2 MTS assay
2.11 \textit{In vivo} experiments
   2.11.1 Animals
   2.11.2 Preparation of cells for injection
   2.11.3 Subcutaneous prostate cancer model
      2.11.3.1 Measurement of subcutaneous tumours
   2.11.4 Orthotopic prostate cancer model
   2.11.5 Intracardiac metastasis model
   2.11.6 Intravenous metastasis model
   2.11.7 Bioluminescent imaging
   2.11.8 Blood glucose measurement
   2.11.9 Removal of tumours
   2.11.10 Serum collection
2.12 Statistical analysis

Chapter 3: Involvement of the IGF pathway in prostate cancer
3.1 Introduction
3.2 Results
   3.2.1 IGF IR expression in prostate cancer TMAs
   3.2.2 Plasma IGF I and PAPP-A levels in BPH and prostate cancer patients
   3.2.3 Expression of the IGF pathway in PC-3M-luc2 cells
   3.2.4 Cell proliferation and VEGF$_{165}$ expression in PC-3M-luc2 cells
   3.2.5 Activation of the IGF signalling pathway by IGF I in PC-3M-luc2 cells
   3.2.6 IGF IR forms heterodimers with the EGFR and IR in PC-3M-luc2 cells
   3.2.7 Proliferation of PC-3M-luc2 cells in response to EGF
   3.2.8 EGFR and IGF IR activation following EGF treatment in PC-3M-luc2 cells
3.3 Discussion

Chapter 4: Purification and \textit{in vitro} evaluation of protease resistant IGFBP4 in prostate cancer cells
4.1 Introduction
4.2 Results
   4.2.1 Isolation of dB4-expressing HEK 293 single cells clones
4.2.2 Purification of dBP4 from HEK 293 single cell clones 106
4.2.3 Cleavage of dBP4 by PAPP-A and PSA 110
4.2.4 IGF binding to dBP4 110
4.2.5 Effect of tyrosine kinase inhibitors on PC-3M-luc2 cell proliferation 115
4.2.6 Effect of dBP4 on PC-3M-luc2 cell proliferation 117
4.2.7 Effect of dBP4 on angiogenesis in human endothelial cells 117

4.3 Discussion 123

Chapter 5: In vivo effect of inducible vector expressed protease resistant IGFBP 4 on prostate cancer growth 127

5.1 Introduction 128
5.2 Results 132
  5.2.1 Cloning of dBP4 into pLVX Tight Puro lentiviral vector 132
  5.2.2 Transduction of PC-3M-luc2 and HEK 293 cells with pTet-On Advanced vector 136
  5.2.3 Expression of rtTA and dBP4 in doxycycline-induced expression system 136

5.3 In vivo effect of inducible vector expressed dBP4 in prostate cancer 139
  5.3.1 Expression of dBP4 in a subcutaneous model of prostate cancer 139
  5.3.2 Long-term effect of inducible vector expressed dBP4 in a subcutaneous model of prostate cancer 141
  5.3.3 Effect of inducible vector expressed dBP4 in an orthotopic model of prostate cancer 154
  5.3.4 Effect of inducible vector expressed dBP4 in models of experimental metastasis 165
    5.3.4.1 Effect of inducible vector expressed dBP4 following intracardiac injection of PC-3M-luc2 cells 165
    5.3.4.2 Effect of inducible vector expressed dBP4 following IV injection of PC-3M-luc2 cells 168

5.4 Discussion 178

Chapter 6: Concluding Discussion 187

References 198

Appendices 241
Appendix 1: Vector map of pTriEx4-Neo 242
<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Vector map of pTet-On Advanced</td>
<td>243</td>
</tr>
<tr>
<td>3</td>
<td>Vector map of pLVX Tight Puro</td>
<td>244</td>
</tr>
<tr>
<td>4</td>
<td>Vector map of psPAX2</td>
<td>245</td>
</tr>
<tr>
<td>5</td>
<td>Vector map of pmD2.g</td>
<td>246</td>
</tr>
<tr>
<td>6</td>
<td>Buffer and reagents for Western blotting, FPLC and DNA electrophoresis</td>
<td>247</td>
</tr>
<tr>
<td>7</td>
<td>Representative Bio-Rad DC protein assay standard curve</td>
<td>249</td>
</tr>
<tr>
<td>8</td>
<td>Antibodies used in IHC, IP and Western blotting</td>
<td>250</td>
</tr>
<tr>
<td>9</td>
<td>Representative VEGF&lt;sub&gt;165&lt;/sub&gt; standard curve</td>
<td>254</td>
</tr>
<tr>
<td>10</td>
<td>Representative human IGF I standard curve</td>
<td>255</td>
</tr>
<tr>
<td>11</td>
<td>Representative mouse IGF I standard curve</td>
<td>256</td>
</tr>
<tr>
<td>12</td>
<td>Representative human PAPP-A standard curve</td>
<td>257</td>
</tr>
<tr>
<td>13</td>
<td>Amino acid sequence of protease resistant IGFBP 4</td>
<td>258</td>
</tr>
<tr>
<td>14</td>
<td>Presentations and awards</td>
<td>259</td>
</tr>
</tbody>
</table>
Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree PhD, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original and, to the best of my knowledge, does not breach copyright law and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

Student Number 07209045

Date 17/5/2012
This thesis is dedicated to my parents Thomas and Catherine Kirwan
Acknowledgements

I would firstly like to thank my supervisor Dr. Judy Harmey for giving me the opportunity to undertake a PhD in her group. I would also like to thank my co-supervisor Prof. Elaine Kay Tony O'Grady and Gillian O'Hurley for their help and expertise regarding the clinical aspects of my thesis. I would like give an enormous thank you to Dr. Andrew Kung in Dana Farber Cancer Institute, Boston for giving me the fantastic opportunity to work with him and his lab group in Boston. While I was there, he was a great support and resource for me. In particular, I would like to thank Amanda, Kristen and James for setting up the animal experiments and for having the patience to explain everything to me especially since I had never carried out animal work before. Kristen, a huge thank you for taking over my project when I came back to Ireland and for the continuous updates. So sorry you were given a big project especially when you had just started. Thank you to Prof. William Gallagher and Dr. Radek Zagozdzon for their assistance with the Tet inducible system it really turned my PhD around in a great way. Thank you to Prof. William Watson for providing me with the samples from the PCRC. I would also like to thank Dr. Amanda Tivnan, Dr. Constanze Schadow, Dr. Heidi Daxecler and Dr. Claus Oxvig. I want to thank Prof. John Waddington, Prof. Kevin Nolan and Dr. Helen McVeigh for their continuous support throughout my PhD. I would also like to thank Dr. Maria Morgan for her help with proof reading my thesis. The girls in lab 1, Yvonne, Silvia and Sinead, you have been the greatest support network I could have asked for, you have all become great friends. Thanks for putting up with my rants when things weren’t going so well. To the other 6 HRB scholars, we have finally made it!! Again you guys were another source of support.

A big thank you to my Mum and Dad for all their encouragement and constant interest in my work and thank you to my brothers Paul and Steven. To the three best friends I could ever have asked for Aoife, Mandy and Aoife, you guys made life outside my Ph.D fun especially when times were tough.

Finally a special thanks to my boyfriend Diarmuid, you constantly gave me the encouragement, strength and support I needed. You always reminded me to look on the bright side of life and to stay optimistic no matter what happened, even if you never understood a word of what I was talking about you still listened. Finally I can get a 'real' job as everyone keeps telling me.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenine diphosphate</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl) benzenesulphonyl fluoride</td>
</tr>
<tr>
<td>AI</td>
<td>Androgen independent</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen Response Element</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Tissue Culture</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2 associated death promoter</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma protein-2</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3'-indolyphosphate</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescent imaging</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge couple device</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CXC4</td>
<td>C-X-C chemokine factor 4</td>
</tr>
<tr>
<td>CXC4R</td>
<td>C-X-C chemokine factor 4 receptor</td>
</tr>
<tr>
<td>Cys</td>
<td>Cystine</td>
</tr>
<tr>
<td>dBP4</td>
<td>PAPP-A resistant IGFBP4</td>
</tr>
<tr>
<td>DC</td>
<td>Detergent compatible</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital rectal exam</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithioretilot</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethyldiamine tetracetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EK</td>
<td>Enterokinase</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FDG-PET</td>
<td>Fluorodeoxyglucose - positron emission tomography</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GHRH</td>
<td>Growth hormone releasing hormone</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
</tbody>
</table>
H & E  Haematoxylin & Eosin
HCl  Hydrochloric acid
HEK  Human embryonic kidney
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF  Hypoxia inducible factor
HRP  Horseradish peroxidase
i.p.  Intraperitoneal
IGF  Insulin-like growth factor
IGF R  Insulin-like growth factor receptor
IGFBP  Insulin-like growth factor binding protein
IgG  Immunoglobulin G
IP  Immunoprecipitation
IR  Insulin receptor
iRS-1  insulin receptor substrate-1
IV  Intravenous
IVIS  In vivo imaging system
KCl  Potassium chloride
KO  Knockout
LAF  Laminar airflow hood
LB  Luria Bertani
Leu  Leucine
LID  Liver-specific IGF I-deficient
LMP  Low melting point
LSD  Fisher's least significant difference
Luc  Luciferase
mAb  Monoclonal antibody
MAPK  Mitogen activated protein kinase
MEM  Modified Eagle medium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPCR</td>
<td>Multiplex polymerase chain reaction</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTD</td>
<td>Mean tumour diameter</td>
</tr>
<tr>
<td>MTS</td>
<td>[3-(4,5\text{-dimethylthiazol-2-yl})-5\text{-}(3-carboxymethoxyphenyl)-2\text{-}(4-sulfophenyl)-2H-tetrazolium]</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Sodium Phosphate</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro-blue Tetrazolium</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NMSC</td>
<td>Non melanoma skin cancer</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>ns</td>
<td>Non significant</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung carcinoma</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD SCID Gamma</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>Pregnancy associated plasma protein-A</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
</tbody>
</table>
PI3K
PIN
PMS
POD
PSA
PSMA
PTEN
Puro
RIPA
RNA
RNAi
RT
rtTA
RXR
SCID
SDS
SEM
Ser
Shc
siRNA
SOC
SPECT
TAE
TBST
TGFβ
Thr
TKI
TMA

Phosphatidylinositol 3 kinase
Prostatic intraepithelial neoplasia
Phenazine methosulfate
Peroxidase
Prostate Specific Antigen
Prostate specific membrane antigen
Phosphatase tensin homolog
Puromycin
Radioimmunoprecipitation assay buffer
Ribonucleic acid
Ribonucleic acid interference
Reverse transcription
Reverse transcriptional transactivator
Retinoid X receptor
Severe combined immunodeficiency
Sodium dodecyl sulfate
Standard error of the mean
Serine
Src homology (SH)-2 containing protein
Short interfering ribonucleic acid
Super optimal broth with catabolite repression
Single photon emission tomography
Tris-acetate-EDTA
Tris buffered saline-tween
Transforming growth factor beta
Threonine
Tyrosine kinase inhibitor
Tissue microarray
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNM</td>
<td>Tumour, node, metastasis</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma mouse prostate</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline response element</td>
</tr>
<tr>
<td>tTA</td>
<td>Tetracycline controlled transactivator</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VES</td>
<td>Vitamin E Succinate</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel Lindau</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>Units</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cm²</td>
<td>centimetre squared</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>× g</td>
<td>acceleration due to gravity</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>μM</td>
<td>micromole</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ml</td>
<td>millilitres</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>millimole</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nM</td>
<td>nanomole</td>
</tr>
<tr>
<td>p</td>
<td>photons</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>% w/v</td>
<td>percentage weight per volume in 100 ml</td>
</tr>
<tr>
<td>% v/v</td>
<td>percentage volume per volume in 100 ml</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>sr</td>
<td>steradian</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
</tbody>
</table>
Abstract

The insulin-like growth factor (IGF) pathway consists of two ligands, IGF I and IGF II, their two receptors, the IGF I receptor (IGF IR) and IGF II receptor along with several soluble insulin-like growth factor binding proteins (IGFBPs). The IGFBPs bind to and modify IGF activity. However, proteases such as pregnancy associated plasma protein A (PAPP-A) can cleave IGFBPs, releasing active IGF I to stimulate the IGF IR. Stimulation of the IGF IR leads to activation of the Akt or mitogen-activated protein kinase (MAPK) pathway that can lead to transformation, differentiation, proliferation and angiogenesis. Increased IGF IR expression has been reported in prostate cancer tissue relative to normal or benign tissue. Therefore, targeting the IGF pathway may prove useful in treating prostate cancer.

Tissue microarrays of prostate tumours were stained with an antibody to the IGF IR. IGF IR expression was significantly associated with Gleason grade with IGF IR expression higher in Gleason grade 3 relative to normal/benign prostatic hyperplasia (BPH) tissue and lower in Gleason grade 5 relative to Gleason grade 3 tissue. There was no significant difference in plasma IGF I levels between BPH and prostate cancer patients (Gleason score 5 or Gleason score 7). However, there were significantly higher plasma levels of the IGFBP 4 protease, PAPP-A, in Gleason score 5 patients relative to BPH patients.

PC-3M-luc2 cells are a human androgen independent cell line lacking the androgen receptor. The PC-3M-luc2 cells stably express luciferase allowing them to be imaged in vivo using bioluminescent imaging. Expression of the IGF pathway components was assessed in these cells to ensure the IGF pathway was active. Western blot analysis showed that the PC-3M-luc2 cells expressed the IGF IR which was activated by IGF I. IGFBP 3, IGFBP 4 and PAPP-A were also expressed. PC-3M-luc2 cells were shown to proliferate in response to IGF (E3R) (an analogue of IGF I that cannot be bound by IGFBPs) by bromodeoxyuridine (BrdU) assay. However, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay showed PC-3M-luc2 cells proliferated in response to IGF I. IGF (E3R) also significantly increased vascular endothelial growth factor (VEGF) expression in PC-3M-luc2 cells.

We assessed a PAPP-A resistant mouse IGFBP 4 (dBp4) as a strategy to neutralise the IGF I ligand. dBp4 was developed by mutation of the PAPP-A cleavage site from 119-KHMAKVRD1R5KMK-133 to 119-AAMAVADASAMA-133 and cloned into pTriEx4 Neo plasmid with C-terminal His-tag for expression in human embryonic kidney cells. Purified dBp4 was resistant to PAPP-A cleavage and retained IGF I binding capacity. In vitro, dBp4 significantly inhibited tubule formation by endothelial cells relative to controls or IGF I treated cells suggesting that dBp4 possesses anti-angiogenic properties.

A Tet inducible expression system was used in vivo to test the effect of dBp4 on tumour growth and metastasis in subcutaneous, orthotopic and experimental metastasis
models of prostate cancer. The inducible Tet-On system allowed dBp4 expression to be induced by doxycycline. Following implantation of PC-3M-luc2 dBp4 cells into non-obese diabetic, severe combined immunodeficiency, gamma 2 receptor null (NSG) mice, tumours and metastasis were monitored by bioluminescent imaging and/or caliper measurements. In the subcutaneous tumour model, dBp4-expressing subcutaneous tumours were significantly smaller than controls and in the orthotopic model, bioluminescence was reduced relative to controls. Mice bearing dBp4-expressing subcutaneous tumours survived significantly longer than controls. There was no survival difference between mice bearing dBp4-expressing prostate tumours and controls. IGF I expression within the dBp4-expressing subcutaneous and prostate tumours was decreased relative to controls. Western blot analysis of tumour lysates showed that dBp4 inhibited IGF IR activation in both the subcutaneous and prostate tumours, which may be a consequence of reduced IGF I within the dBp4-expressing tumours. However, activated Akt was only decreased in the subcutaneous tumours relative to controls. As tumours require vasculature in order to supply oxygen and nutrients essential for tumour growth, expression of VEGF was assessed in the subcutaneous tumours. Control tumours expressed VEGF164/165, which was not expressed in the dBp4-expressing subcutaneous tumours.

dBp4 expression inhibited tumour growth in both subcutaneous and orthotopic prostate cancer models. As dBp4 expression inhibited endothelial cell tubule formation and abolished VEGF164/165 expression within PC-3M-luc2 tumours growing subcutaneously, this suggests that dBp4 has either anti-angiogenic or anti-proliferative properties and may therefore have value as a therapeutic for prostate cancer.
Chapter 1

General Introduction
1.0 Introduction

1.1 Cancer
In 2008, 12.7 million new cancer cases and 7.6 million cancer deaths occurred worldwide. The number of new cancer cases ranged from 3.7 million in Eastern Asia to 1,800 in Micronesia/Polynesia. In men, the incidence of cancer is high in Northern America, Australia/New Zealand and in Northern and Western Europe because of the high rates of prostate cancer in these regions. The regions with the highest incidence rates in females are Northern America, Australia/New Zealand, Northern and Western Europe because of the high rates of breast cancer in these regions (GLOBOCAN2008 Cancer Fact Sheet, 2008).

Between 2005 and 2007, 27,023 new cases of cancer were diagnosed in Ireland annually. The most common cancer excluding non-melanoma skin cancer (NMSC) was prostate cancer (2,462), followed by breast cancer (2,335), colorectal cancer (2,156) and lung cancer (1,810). Breast cancer was the most common cancer in women apart from NMSC and prostate cancer was the most common in men. In 2006, Ireland had the second highest cancer rates in Europe, with lung cancer being the major cause of cancer related mortality with over 1,600 deaths registered (National Cancer Registry, Cancer in Ireland 1994 - 2007 (2009)). From this data, it is clear that cancer is a major problem affecting the Irish population. Cancer incidence is rising yearly; this may be due to an increase in screening programmes available to the public. As the life expectancy of the population increases, mortality rates from prostate cancer are also expected to rise.

1.2 Development of cancer
Carcinogenesis is the term used to describe cancer growth or development. Irrespective of the cause of cancer, initiation of the disease involves transformation of healthy cells. This causes a change in the cells' genetic material, priming the cells to become cancerous. This change can occur spontaneously as a consequence of inherited mutations or by exposure to carcinogens. Tumours develop when a mutation occurs in key genes that are important in control of cell growth (Whang et al., 1998). Key genes involved in cancer formation are termed oncogenes and tumour suppressor genes (Pesche et al., 1998). C-myc is one such oncogene that regulates cell proliferation and is overexpressed in prostate cancer (Ellwood-Yen et al., 2003). Tumour suppressor phosphatase and tensin homolog (PTEN) is commonly mutated or lost in prostate cancer, leading to overactivation of the Akt pathway (Mc Menamin et al., 1999). Once the initial transformation event has occurred, other co-factors such as growth factors and chemicals promote the growth of the cancerous cells (Liotta and Kohn, 2001).

Tumours can be broadly classed as either benign or malignant. Benign tumours grow slowly, are not aggressive, do not invade surrounding tissues and do not metastasise.
In contrast, malignant tumours grow rapidly, are aggressive, invade surrounding tissues and cause metastasis. Once the tumour cells invade local tissue they can access and circulate in, the lymphatic or circulation system and cause metastasis in distant organs (Fidler et al., 2007).

1.3 Prostate Cancer
Prostate cancer is the second most common cancer in men following skin cancer. One in 12 men will be diagnosed with prostate cancer in their lifetime. In 2009, 2,758 men were diagnosed with the disease in Ireland and over 500 deaths occur annually. The number of cases of prostate cancer is rising annually (3 %) due to an increase in the life expectancy of the population. One of the largest changes between 1994 and 2009 was in the 55 - 64 year age group, with the number of cases increasing from 134 cases in 1994 to 859 in 2009 (15 % increase annually). However, the largest relative increase was seen in younger men less than 55 years old (19 % annually). The number of men receiving prostate specific antigen (PSA) tests increased 5 fold from 1994 - 2004 and this may explain the increase in prostate cancer rates in young men (National Cancer Registry, Cancer trend No.3. Recent trends in prostate cancer (2010)).

1.3.1 Risk factors for prostate cancer
Risk factors increase an individual's likelihood of developing a certain disease. In the case of prostate cancer, the main risk factors are age, family history, race and diet.

1.3.1.1 Age
Age is the strongest risk factor for prostate cancer. Prostate cancer is less common in men under the age of 40, but the risk of developing the disease increases in men over 50 years of age. The link with age may be due to an increase in oxidative stress seen with ageing. Ageing is associated with many metabolic disorders (Furukawa et al., 2004) and various cancers (Szatrowski and Nathan, 1991). An increase in oxidative stress may explain the increased incidence of prostate cancer with advancing age. Studies show an altered pro-oxidant:anti-oxidant status in prostatic tissue, with decreased anti-oxidant enzymes observed in prostatic intraepithelial neoplasia (PIN) and prostate cancer (Bostwick et al., 2000). In vitro, LNCaP cells, an androgen dependent human prostate cancer cell line, treated with a synthetic androgen (R1881) showed an increase in oxygen consumption and catalase activity relative to untreated controls (60% and 40%, respectively). R1881 treatment resulted in a decrease in intracellular glutathione concentrations and an increase in γ-glutamyl transpeptidase activity in LNCaP cells relative to untreated controls suggesting
that androgen receptor signalling may alter the pro-oxidant:anti-oxidant balance of prostatic cells (Ripple et al., 1997).

1.3.1.2 Family History
Family history is associated with an increased risk of prostate cancer. One study has shown that men with first degree relatives (father or brother) with prostate cancer had double the risk of developing prostate cancer and of early onset disease (Ahn et al., 2008). Men with a positive family history were four times more likely to have advanced stage disease when diagnosed (Ahn et al., 2008). Evidence also suggests that men are at a 70% higher risk of developing prostate cancer if there is a family history of breast and/or ovarian cancer, indicating a possible genetic involvement (Cerhan et al., 1999). As family history is a strong risk factor for prostate cancer, individuals with a family history of the disease should be screened early on to prevent diagnosis at an advanced stage.

1.3.1.3 Race/Ethnicity
African-American men have a higher risk of prostate cancer development relative to non African-Americans (Thompson et al., 2006). African-American men also have a higher risk of high-grade disease (odds ratio: 2.61). Asian countries such as Japan have the lowest incidence and risk of prostate cancer. The difference in prostate cancer risk between the two countries may be attributed to diet as well as genetic background, with higher consumption of red meat in the USA compared with fish in Japan. Lack of access to healthcare and health insurance was another factor involved in the higher rates of prostate cancer in African-American men (Jones et al., 2008).

1.3.1.4 Diet
The involvement of a high fat diet in prostate cancer risk is conflicting, with pre-clinical models showing that energy intake may be more important than dietary fat (Mukherjee et al., 1999). However, a correlation was established between increased body weight and increased risk of prostate cancer (Andersson et al., 1997). Obesity has a strong link with prostate cancer risk; in particular men with a body mass index (BMI) greater than 30 are at a higher risk of high grade prostate cancer (Gong et al., 2006). This illustrates the importance of physical activity and weight control in prostate cancer development.

Vitamin E (α-tocopherol) is an anti-oxidant. Long-term supplementation with Vitamin E reduced prostate cancer incidence and mortality in smokers (Heinonen et al., 1998). In lung cancer, female A/J mice were injected with urethane to initiate tumour development, Vitamin E inhibited cell proliferation in the promotion phase of lung tumourigenesis via blockade of the ERK pathway (Yano et al., 2000). A derivative of Vitamin E called Vitamin E
succinate (VES) inhibited the growth of androgen dependent LNCaP prostate cancer cells \textit{in vitro} by suppressing androgen receptor function and expression of PSA (Zhang \textit{et al.}, 2002b).

Low levels of Vitamin D are hypothesised to be associated with increased cancer incidence and mortality in men (Giovannucci \textit{et al.}, 2006). Gene polymorphisms in the Vitamin D receptor (VDR) are thought to be associated with prostate cancer risk (Habuchi \textit{et al.}, 2000). Men with a particular polymorphism (BsmI) in the VDR were protected against prostate cancer and benign prostatic hyperplasia (BPH) in a Japanese cohort (Habuchi \textit{et al.}, 2000). However, further studies are needed to verify the correlation with age and other racial groups. Prostate cancer cells express the VDR (Skowronski \textit{et al.}, 1993) and \textit{in vitro}, 1,25 dihydroxy vitamin D$_3$, a metabolite of Vitamin D, inhibited the growth of androgen dependent LNCaP prostate cancer cells (Blutt \textit{et al.}, 1997). By decreasing calorie intake and increasing Vitamin D and E intake prostate cancer risk may be decreased substantially (Dunn \textit{et al.}, 1997, John \textit{et al.}, 2004, Heinonen \textit{et al.}, 1998). However, changing dietary habits does not completely rule out development of prostate cancer as other risk factors previously mentioned also play a role.

\subsection*{1.3.2 Treatment of prostate cancer}
Stage, grade, age, size of tumour, PSA levels and general health of the patient are all taken into account when deciding on treatment options. The most commonly available treatment options include active surveillance, surgery, external beam radiotherapy, brachytherapy, hormone therapy and chemotherapy.

\subsubsection*{1.3.2.1 Active Surveillance}
In early stage prostate cancer, the tumour is usually slow growing. Some specialists may wait to see if the cancer is growing before deciding on a treatment. Advanced age may also be a clinical indicator for active surveillance. Active surveillance is also recommended if the cancer is small and localised to one area within the prostate and not causing any symptoms. Active surveillance involves regular check ups to monitor PSA levels with rectal examination and prostate biopsy carried out if needed. If PSA levels increase dramatically and symptoms begin to occur, other treatment options are then considered (Irish Cancer Society (2010)).

\subsubsection*{1.3.2.2 Surgery}
In 2007, 27 \% of prostate cancer patients in Ireland had a radical prostatectomy (National Cancer Registry, Cancer trend No.3. Recent trends in prostate cancer (2010)). A radical prostatectomy involves removal of the whole prostate. This can occur only if the tumour is localised within the prostate. This procedure can be carried out by open surgery or “key-
hole" surgery. The main side effects associated with radical prostatectomy are incontinence and impotence (Irish Cancer Society (2010)).

1.3.2.3 Radiation Therapy
The number of prostate cancer patients receiving radiotherapy has increased from 19 % in 1996 to 41 % in 2007 (National Cancer Registry, Cancer trend No.3. Recent trends in prostate cancer (2010)). Radiation therapy damages the DNA within a cell when it is dividing and as cancerous cells divide more rapidly than healthy cells, damage to healthy cells is reduced. This therapy is useful in the treatment of early stage prostate cancer and it is used if the cancer cannot be completely removed or a recurrence occurs. There are two types of radiation therapy available, external beam radiotherapy and brachytherapy.

1.3.2.3.1 External Beam Radiotherapy
External beam radiotherapy uses high energy X-rays to destroy cancer cells and cause as little harm to normal cells as possible. Side effects of this therapy include impotence, incontinence, tiredness, skin irritation, diarrhoea, upset stomach and burning urination (Prostate Cancer Treatment Guide, (2010)).

1.3.2.3.2 Brachytherapy
Brachytherapy is radiation therapy given within the body. Small radioactive pellets are placed into the tumour. Radiation is emitted slowly over time, but this therapy is only useful if the tumour is confined within the prostate. Commonly used radioactive isotopes are iodine-125 and palladium-103. Side effects include impotence, incontinence and blood in the urine (Prostate Cancer Treatment Guide, (2010)).

1.3.2.4 Chemotherapy
Chemotherapy is used in advanced prostate cancer with bone metastasis (Prostate Treatment Guide, (2010)). It is ineffective against dormant metastases that are not proliferating. Chemotherapy is given intravenously and the most commonly used drugs are paclitaxel (Morris, 2006) and docetaxel (Petrylak, 2006). Similar to radiation therapy these chemotherapeutics specifically target dividing cells in the body. As chemotherapy is not directed at any particular organ, chemotherapeutics kill normal proliferating cells, as well as cancer cells. Normal cells affected include those of hair follicles, gastro-intestinal (GI) tract and bone marrow resulting in the side effects associated with chemotherapy - nausea, vomiting, hair loss, immunosuppression and infertility (Prostate Treatment Guide, (2010)).
1.3.2.5 Hormone Therapy

The male hormone testosterone promotes the growth of androgen receptor positive prostate cancer, hence blocking its activity inhibits androgen dependent tumour growth (Ross et al., 1998). Hormone therapy can be given before surgery with the aim of shrinking primary tumours to allow surgical excision or as an adjuvant alongside another therapy (Aus et al., 2002, Bolla et al., 2002) and also in metastatic prostate cancer (Messing et al., 1999). An orchidectomy can be carried out, which removes the testes thus removing the source of androgen production. Other hormone therapies include anti-androgens (flutamide) and luteinising hormone releasing hormone agonists (Tolis et al., 1982). The percentage of prostate cancer patients receiving hormone therapy has decreased from 46% in 2000 to 35% in 2007. (National Cancer Registry, Cancer trend No.3. Recent trends in prostate cancer (2010)). Hormone therapies can be taken in tablet form as an outpatient, which avoids hospital visits and improves the patient's quality of life. This may explain the apparent decrease in patient's receiving hormone therapy, as all cases are not recorded by the National Cancer Registry (National Cancer Registry, Cancer trend No.3. Recent trends in prostate cancer (2010)). However, inhibition of testosterone production can lead to unwanted side effects: hot flashes, memory loss, erectile dysfunction and osteoporosis. Blocking testosterone production does not eradicate the tumour, but prevents disease progression. Within 18 - 24 months of hormone therapy, the cancer may become resistant (Rau et al., 2005) particularly in advanced disease. This is known as hormone refractory or androgen independent (AI) disease.

1.4 Androgen independent prostate cancer

Androgens are involved in normal prostate growth as well as prostate cancer cell growth and proliferation. Androgens mediate their effects via the androgen receptor (AR) to promote growth and survival (Xu et al., 2006). Testosterone is the main circulating androgen that is primarily secreted by the testes. Testosterone enters the cell and is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT is a more active hormone with a higher affinity for the AR than testosterone (K_D=4.4 nM vs. 10.9 nM, respectively) (Deslypere et al., 1992, Kumar et al., 1999). The AR is a member of the steroid-thyroid-retinoid receptor superfamily. It contains an activating domain, a ligand-binding domain and a DNA-binding domain. In the resting state, the AR is bound to heat shock proteins that prevent DNA binding. Upon binding of DHT, a conformational change in the AR leads to dissociation from the heat shock proteins and receptor phosphorylation occurs (Nazareth and Weigel, 1996). The ligand induced conformational change allows the AR to form homodimers that bind to androgen response elements (ARE) in promoter regions of target genes. Once the AR is bound to the DNA co-activators, co-regulatory proteins or co-
repressors are recruited. During androgen independent progression, prostate cancer cells develop numerous cellular pathways to survive in an androgen depleted environment. The main mechanisms are hypersensitive pathway, a promiscuous pathway, an outlaw pathway and a bypass pathway (Pienta and Bradley, 2006).

1.4.1 Hypersensitive Pathway
One mechanism by which prostate cancer cells circumvent androgen blockade is by developing the ability to respond to low levels of androgen. AR amplification has been observed in AI prostate cancer, with a 6 fold higher expression of AR in AI prostate cancer patients relative to androgen dependent patients (Linja et al., 2001). It has also been shown that prostate cancer cells (CWR22, LNCaP, LuCap23, LuCap35, LuCap41, LAPC-4 and LAPC-9) grown subcutaneously in castrated nude mice (androgen-depleted) required 80 % lower concentrations of androgen relative to the same cells growing in non-castrated nude mice (androgen sensitive) (Chen et al., 2004). Alternatively, increasing AR expression allows for enhanced ligand binding even in an androgen-depleted environment.

A second mechanism is increased sensitivity of the AR to androgens. Gregory et al (2001) used androgen dependent LNCaP cells and cell lines derived from recurrent prostate cancer tumours to show that in the absence of androgen the AR was more stable in the recurrent tumour relative to the LNCaP cells and this stability was associated with hypersensitivity to DHT. The concentration of DHT needed to stimulate growth in the recurrent tumour cell lines was 4 orders of magnitude lower than that needed for the androgen dependent LNCaP cells (Gregory et al., 2001).

The third hypersensitive mechanism proposed is increased local production of androgens by prostate cancer cells. This most likely occurs by increased rate of conversion of testosterone to DHT by 5α-reductase. Several studies support this mechanism. One study showed that ethnic groups with higher levels of 5α-reductase activity had a higher rate of prostate cancer (Makridakis et al., 1997). Also, genes involved in steroid biosynthesis have been found to be overexpressed in recurrent prostate tumours (Holzbeierlein et al., 2004).

1.4.2 Promiscuous Pathway
The AR is specifically activated by testosterone and DHT, but mutations in the AR broaden its specificity. The majority of the AR mutations are present in the ligand-binding domains (McPhaul et al., 1992). These mutations allow non-androgenic steroid molecules, usually in circulation within the body and anti-androgens to activate the AR (Miyamoto et al., 1998, Zhao et al., 2000). Mutations in the AR are more frequent in metastatic AI prostate cancer (Taplin et al., 1995) and mutations in the AR are involved in the progression of prostate
cancer to androgen independence (Tilley et al., 1996). An anti-androgen syndrome has
been associated with a mutated AR. Flutamide, a commonly used anti-androgen can
activate the mutated AR (Miyamoto et al., 1998). Metastatic prostate cancer patients
(n=107) given flutamide showed a progression in disease, however upon withdrawal of
flutamide, PSA levels decreased by 50-80 % (Small and Srinivas, 1995). Androgen ablation
may lead to clonal selection of prostate cancer cells harbouring the AR mutations thus
conferring a growth advantage. Alterations in proteins that act together with the AR, such as
co-activators or co-repressors, which act as intermediaries between the AR and
transcriptional machinery, have been observed in AI disease. An increase in co-activator
levels such as cell division cycle 25b has been shown in higher Gleason score tumours
relative to lower Gleason score tumours (Comuzzi et al., 2003, Ngan et al., 2003).

1.4.3 Outlaw Pathway
The outlaw pathway involves activation of the AR independent of androgens. Growth and
proliferation of tumour cells is no longer under androgen control. Growth factors including
insulin-like growth factor I (IGF I) and epidermal growth factor (EGF) directly phosphorylate
and activate the AR (Culig et al., 1994). Androgen dependent genes can be activated by de-
regulated signal transduction pathways. The HER2/Neu receptor is a member of the EGF
receptor family and overexpression of this receptor in androgen dependent LAPC-4 prostate
cancer cells converted them to AI cells. HER2/Neu expression led to activation of androgen
receptor dependent genes in the absence of ligand (Craft et al., 1999). PTEN, a tumour
suppressor, which negatively regulates Akt activity (Stambolic et al., 1998) is often lost or
mutated in advanced prostate cancer (Li et al., 1997, Mc Menamin et al., 1999). Akt has
been implicated in tumourigenesis due to its anti-apoptotic activity. In Zhou et al, (2000b)
Akt phosphorylated and inactivated the pro-apoptotic proteins Bcl-2 associated death
promoter (BAD) and procaspase-9 in hybrid motor neuron 1 cells. Therefore, by blocking Akt
activity, PTEN allows cells to undergo apoptosis. Therefore, loss of PTEN results in
resistance to apoptosis (Stambolic et al., 1998)). Prostate cancer cells that have lost PTEN
function have increased Akt activity (Graff et al., 2000). Graff et al (2000) established
androgen independent cell lines (LNAI) from xenografts of LNCaP cells grown
subcutaneously in castrated male nude mice. In that study, increased Akt activity was
observed in the AI LNAI cells relative to the parental androgen dependent LNCaP cells.
High levels of phospho Akt in prostate cancer is a strong predictor of biochemical recurrence
(Ayala et al., 2004). Akt may also be an alternative pathway by which HER2/Neu activates
the AR signalling as the HER2/Neu receptor activates Akt (Zhou et al., 2000).
1.4.4 Bypass Pathway

This mechanism does not require the AR and its signalling cascade allowing AI prostate cancer cells to proliferate independently of ligand mediated AR activation. The most studied bypass pathway involves modulation of apoptosis. In androgen dependent cells the AR stimulates cell proliferation and survival, therefore depletion of androgens leads to death of the androgen dependent cells. However, in AI cells, anti-apoptotic molecules are frequently up-regulated. Bcl-2 levels are higher in AI prostate carcinomas relative to androgen dependent prostate carcinomas, suggesting that Bcl-2 is involved in the progression from androgen dependent to AI disease (Mc Donnell et al., 1992). Overexpression of Bcl-2 protects androgen dependent LNCaP prostate cancer cells from apoptosis in vitro and in a subcutaneous nude mouse model. LNCaP/bcl-2 tumours grew in castrated mice compared with parental LNCaP cells, which did not form tumours in castrated mice (Raffo et al., 1995). However, overexpression of Bcl-2 is not essential for the development of AI prostate cancer (Furuya et al., 1996) suggesting a role for other bypass mechanisms or substitution by one of the other pathways mentioned.

1.5 Metastasis

Metastasis occurs when cancer spreads from where it first arose (primary tumour) to other organs in the body. Metastases that arise from the primary tumour prior to removal can lie dormant before they are detected, making them difficult to treat. Metastasis occurs more often in advanced cancers where cells become more invasive and aggressive. The formation of metastases is a highly selective process consisting of many interrelated steps. In order for cancer cells to metastasise they must first disseminate from the primary tumour. Some tumour cells detach from neighbouring cells and then invade the extracellular matrix, which requires motility, migration of the tumour cells and breakdown of the extracellular matrix (Hasan et al., 1998, Hazan et al., 2000, Webber et al., 1995). Tumour cells can then penetrate blood vessels and the lymphatic system thus entering the circulation. While circulating in the blood, tumour cells must evade immune cells and the mechanical stress of blood flow. Tumour cells that survive will leave the blood circulation and develop a secondary tumour at another site. This complex process involves factors and events such as invasion, migration, angiogenesis, interaction between tumour cells and the microenvironment (Fidler et al., 2007). Microenvironment is important in establishment of metastasis, which was first shown by Stephen Paget (1889) - The 'seed and soil hypothesis'. He proposed that the tumour cell (seed) has a specific affinity for a particular organ (soil) and the metastases would only grow if they were compatible (Paget, 1889).

The role of microenvironment in cancer involves the host tissue participating in the progression of the disease, such as secreting certain growth factors which can enhance
migration, proliferation and survival of tumour cells (Liotta and Kohn, 2001). Interaction with the host’s microenvironment results in phenotypic differences between cells from the primary tumour and cells from the metastasis. Cells that form the metastasis have encountered many different stimuli, which can make them more aggressive than the primary tumour cells.

One factor which contributes to metastasis is hypoxia, which develops as the tumour grows. Hypoxia up-regulates a transcription factor called hypoxia inducible factor (HIF 1α), which then activates vascular endothelial growth factor (VEGF) and other hypoxia regulated genes. Under normoxia HIF 1α is degraded by an enzyme called VHL (Von Hippel Lindau), but in many cancers this enzyme is mutated resulting in de-regulated expression of HIF 1α (Gnarra et al., 1994). It has been reported that in breast cancer cells, (DU-4475, MCF-7, T-47D, MDA-MB-468, MDA-MB-361, MDA-MB-231,) normal VHL decreased CXCR4 receptor expression (chemokine receptor). Binding of this receptor to its ligand (CXC4), allows migrating cells to navigate to specific organs where there are also high levels of the ligand (Muller et al., 2001).

1.5.1 Bone metastasis
Bone is the most common metastatic site of prostate cancer, with 90% of patients with advanced prostate cancer having skeletal metastasis (Bubendorf et al., 2000). Skeletal metastases result in complications that affect the patients quality of life such as bone pain, impaired mobility and spinal cord compression. Certain factors account for the frequency of bone metastasis in prostate cancer. The blood supply to the prostate may facilitate dissemination of tumour cells to certain bone types. A venous plexus surrounds the prostate and this connects to the venous drainage in the spine. This collection of veins may potentially be one of the reasons why lumbrosacral metastases are common in advanced prostate cancer (Batson, 1940). However, anatomical explanation does not account for other areas of bone involved in bone metastasis. As previously mentioned the ‘seed and soil hypothesis’ may provide more information about the formation of bone metastases. Tumour cells express adhesion molecules that allow them to bind to bone marrow stromal cells. In particular αvβ3 integrin is highly expressed in androgen independent PC-3 cells and it may play a role in the attachment of tumour cells to bone cells (Zheng et al., 1999). Bone itself is a rich source of growth factors including IGF I, transforming growth factor β (TGF β), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (Hauschka et al., 1986), all of which can stimulate growth of prostate cancer cells. In prostate cancer, osteoblastic lesions are commonly seen (Hall et al., 2005). Osteoblasts are bone-forming cells. Osteoblastic bone metastases result in an imbalance between bone resorption and bone formation, the latter being favoured. Prostate cancer cells secrete numerous factors
that stimulate osteoblasts, such as IGFs, VEGF, endothelin-1 (ET-1), urokinase plasminogen activator (uPA) and PSA (Achbarou et al., 1994, Granchi et al., 2001, Kawada et al., 2006, Wang and Klein, 2007, Webber et al., 1995). Plasma levels of IGFBP 3 are lower in prostate cancer patients with bone metastases relative to controls and IGFBP 3 levels were an independent predictor of biochemical progression (Shariat et al., 2002). VEGF promotes bone formation by directly activating osteoblasts and facilitating angiogenesis (Ackerstaff et al., 2004). Evidence suggests ET-1, a potent vasoconstrictor, is a central mediator of osteoblastic metastasis (Yin et al., 2003). Serum levels of ET-1 are increased in prostate cancer patients with osteoblastic lesions relative to controls or organ confined prostate cancer patients (Nelson et al., 1995). ET-1 mediates its effects via the ET	extsubscript{A} receptor. Atrasentan, an ET	extsubscript{A} receptor antagonist was used in a clinical trial in Al metastatic prostate cancer. Atrasentan delayed PSA progression, attenuated acid phosphatase and reduced skeletal morbidity (Carducci et al., 2003). Proteases such as uPA and PSA are also implicated in osteoblastic bone metastasis. uPA is produced by prostate cancer cells and overproduction of uPA in Mat LyLu rat prostate carcinoma cells following intracardiac injection (Copenhagen rats) led to increased skeletal metastases relative to controls (Achbarou et al., 1994). uPA can also cleave IGFBP 3 resulting in an increase in free IGF I (Bang and Fielder, 1997). PSA is a kallikrein serine proteinase secreted by prostate cancer cells. Like uPA, PSA can also cleave IGFBPs (IGFBP 3, 4 and 5) (Rehault et al., 2001) enhancing the effects of IGF I. While osteolytic lesions are more commonly seen in metastatic breast cancer patients (Park et al., 2007), metastatic prostate cancer patients can also develop osteolytic lesions. Osteolytic bone metastases involve destruction and resorption of bone with the involvement of osteoclasts. Elevated serum levels of bone resorption markers such as alkaline phosphatase are seen in prostate cancer patients and can be used as marker for the presence of bone metastasis (Jung et al., 2004).

In prostate cancer and many other cancers, the major clinical challenge is metastasis as the primary tumour can normally be effectively treated by surgery, chemotherapy, radiotherapy or a combination of these therapies. Treatment options for metastatic disease are limited and commonly involve radiation therapy, chemotherapy and bisphosphonates. Treatment of PC-3, DU145 and LNCaP cells with pamidronate or zoledronate significantly reduced the growth of all three cell lines in vitro (Lee et al., 2001). Long-term treatment with zoledronic acid reduced the incidence of skeletal related events (pathologic fracture, spinal cord compression, radiation therapy or surgery to bone) in Al metastatic prostate cancer patients compared with placebo controls (Saad et al., 2004). Bisphosphonates block bone resorption when administered to breast cancer patients at a high risk of bone metastasis. Administration of the bisphosphonate, clodronate, decreased the incidence of bone metastasis along with the death rate in high risk breast cancer patients (Powles et al., 2004).
As metastases are heterogeneous from organ to organ and tissue specific, it makes it difficult to develop a therapy that will target all metastases. Therapies targeting growth factors and proteases present either on the tumour cells or in the microenvironment may be of benefit in inhibiting the formation of metastases.

1.6 Insulin-like growth factor (IGF) pathway

The IGF pathway is important in cell growth, proliferation and differentiation (Coolican et al., 1997). The two ligands, IGF I and IGF II, are single chain polypeptides and have 62 % homology in their amino acid sequences; their structure also resembles that of proinsulin (Rinderknecht and Humbel, 1978). The main production sites for both ligands are the liver and bone marrow. IGF I is a potent mitogen for a number of cells and it exerts its mitogenic activity by increasing DNA synthesis (Dufourny et al., 1997). IGF I also inhibits apoptosis by stimulating the expression of Bcl proteins which block the apoptosis pathway (Parrizas and Leroith, 1997). IGF II is thought to play a less important role in postnatal growth than IGF I. IGF II is crucial during embryonic and foetal growth (Chen et al., 2010). IGF I levels change with age as levels increase from birth to puberty and then decrease with age. However, IGF II levels remain stable after puberty. The actions of both IGF I and IGF II are mediated through the insulin-like growth factor I receptor (IGF IR); IGF II can also interact with the IGF II R. IGF I has a 15-fold higher binding affinity for the IGF IR than IGF II (Germain-Lee et al., 1992). The IGF IR and IGF IIR are glycoproteins expressed on the cell surface. Moreover, these receptors differ structurally and functionally. The IGF IR is a receptor tyrosine kinase consisting of two identical α subunits and two identical β subunits. The α subunits are located extracellularly forming the ligand-binding domain and the β subunits are located intracellularly forming the tyrosine kinase domain. The IGF IR shares 60 % homology with the insulin receptor (IR) (Ullrich et al., 1986). Hybrid receptors also form composed of one α subunit and one β subunit of the IGF IR and one α subunit and one β subunit of the IR, its binding affinity for IGF I higher than that for insulin. Therefore, hybrid receptors are thought to function predominantly as an IGF I receptor (Johansson and Arnqvist, 2006).

The IGF IIR is monomeric, has no tyrosine kinase activity and binds only IGF II (Morgan et al., 1987, Oates et al., 1998). Binding of IGF II to IGF IIR leads to degradation of IGF II, therefore the IGF IIR acts an antagonist to IGF II reducing its biological activity (Oates et al., 1998). Ligand binding to the IGF IR leads to a conformational change resulting in autophosphorylation of tyrosine residues located on the β subunit, allowing docking of adaptor proteins, insulin receptor substrate 1 (IRS 1) and Src homology (SH)-2 containing protein (Shc) (Tartare-Deckert et al., 1995). The IGF IR activates two signal transduction pathways, the RAS mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3 kinase (PI3K)/Akt pathway (Valentinis et al., 1999). Activation of the MAPK and PI3K/Akt
pathway results in activation of anti-apoptotic pathways, cell proliferation and differentiation (Gao et al., 2000, Ge et al., 2007, Párrizas et al., 1997b). Figure 1.1 summarises the IGF pathway components.

1.6.1 Insulin-like growth factor binding proteins (IGFBPs)
Several insulin-like growth factor binding proteins (IGFBPs) regulate IGF bioavailability and activity. IGFBPs share some structural homology, which is highly conserved. A conserved amino terminal (N-terminal) domain contains disulfide bonds and the conserved carboxy terminal (C-terminal) is cysteine rich. IGF binding occurs at the N terminal, but the C terminal is also necessary for IGF binding (Qin et al., 1998). The central domain is not conserved and contains sites for post translational modification and proteolytic cleavage (Conover et al., 1995a). The major role of IGFBPs is to transport IGFs, protect IGFs from degradation and regulate their interaction with the IGF IR. IGFBPs have a higher affinity for IGFs than the IGF IR. Therefore, IGFBP binding to IGFs can inhibit activation of IGF IR (Gockerman et al., 1995).

The majority of IGF I in circulation is bound to IGFBP 3 in a complex with an acid labile subunit (ALS), which acts as a reservoir for IGF I and increases the half life of IGF I within the body (Payet et al., 2004). IGFBPs have IGF dependent and independent effects, which inhibit or enhance the actions of IGFs. IGFBP 1, IGFBP 2 and IGFBP 5 have been shown to have IGF independent roles effecting cell migration, apoptosis and cell growth (Chesik et al., 2010, Frommer et al., 2006, Tripathi et al., 2009). IGFBP 3 also exerts IGF independent effects. One study discovered an IGFBP 3 death receptor which binds IGFBP 3 and induced apoptosis in breast (MDA-231) and prostate cancer (M12) cells implanted subcutaneously in female athymic mice (Ingermann et al., 2010). Unlike the other IGFBPs, IGFBP 4 and IGFBP 6 do not enhance IGF activity. IGFBP 4 exclusively inhibits IGFs. It exists in two forms, non-glycosylated (24 kDa) and N-glycosylated (28 kDa). IGFBP 4 binds IGF I and IGF II with equal affinity (Kiefer et al., 1992). Local administration of IGFBP 4 in mouse bone inhibits bone formation (Miyakoshi et al., 1999). Overexpression of IGFBP 4 in smooth muscle cells of transgenic mice using a smooth muscle α-actin-IGFBP 4 fusion gene induced smooth muscle hypoplasia (Wang et al., 1998). This indicates that IGFBP 4 inhibits IGF I actions. IGFBP 4 proteolysis is important in regulating IGF I bioavailability. PSA cleaves IGFBP 3, IGFBP 4 and IGFBP 5 (Rehault et al., 2001) releasing active IGF I. The metalloproteinase, pregnancy-associated plasma protein A (PAPP-A), is the main protease involved in IGFBP 4 cleavage (Boldt and Conover, 2007). PAPP-A cleaves IGFBP 4 into two fragments of 14 kDa and 18 kDa with reduced affinity for IGF I (Conover et al., 1995a). Regulation of IGFBP proteolysis remains poorly understood. It has been shown that IGFs modulate IGFBP degradation, suggesting IGFs work in an autocrine manner to regulate
their own action (Angelloz-Nicoud and Binoux, 1995). Due to the importance of the IGF pathway in cell growth and differentiation, deregulation of this pathway leads to aberrant growth of cells, which is seen in cancer. The involvement of the IGF pathway in numerous cancers including prostate cancer has been well established (Browne et al., 2011, Chan et al., 2002).

1.6.2 IGF pathway and prostate cancer

IGF I and IGF II mitogenic effects have been shown in vitro in a wide variety of cell lines including prostate and breast cancer cell lines (Connolly and Rose, 1994, Stewart et al., 1990). In vivo experiments in transgenic mice show that overexpression of IGF I under the control of the bovine keratin 5 promoter or IGF II under control of mouse mammary tumour virus promoter increased the likelihood of spontaneous tumour development in certain tissues such as mammary ducts and lungs (De Ostrovich et al., 2008, Moorehead et al., 2003). The IGF IR is involved in transformation and maintenance of a transformed phenotype (Kaleko et al., 1990). Constitutively active IGF IR expression in normal mammary epithelial cells caused transformation of cells and epithelial to mesenchymal transition (EMT) in vitro (Kim et al., 2007).

The IGF IR plays an important role in numerous cancers. IGF IR is overexpressed in certain cancers (breast, prostate, sarcoma) and is associated with more aggressive tumours (Xie et al., 1999). As well as IGF IR involvement in primary tumours, the IGF IR has been implicated in metastasis. Neuroblastoma metastasises to bone. Neuroblastoma cell lines constitutively expressing high levels of IGF IR (highly aggressive, IMR32) or transfected with IGF IR (SHEP/IGFR) adhered tightly to bone stromal cells following intratibial injection into female SCID mice relative to non-aggressive cells (SHEP) expressing low levels of IGF IR (Van Golen et al., 2006). Furthermore, inhibition of the IGF IR inhibits metastasis. MDA-MB-435 breast cancer cells transfected with a dominant negative form of the IGF IR showed decreased metastasis formation following implantation into the mammary fat pad of female nude mice relative to vector controls (Dunn et al., 1998). Specifically, in prostate cancer, the IGF IR is overexpressed in primary tumours and remains expressed in metastatic disease (Hellawell et al., 2002). Numerous studies have shown that elevated IGF I levels increase the risk of prostate cancer development. An association between serum IGF I levels and prostate cancer risk was seen in a cohort study (n=210 newly diagnosed prostate cancer patients). A strong association between elevated IGF I levels and prostate cancer risk was observed in men younger than 70 years of age (odds ratio: 2.93) (Wolk et al., 1998). In 1998, results of a prospective case control study of IGF I and IGFBP 3 was reported. Blood samples were taken in 1982 from 14,916 male physicians, frozen and
Figure 1.1 IGF signalling pathway. IGF I/II are bound by IGFBPs in circulation; upon proteolysis of IGFBPs both ligands are free to bind the IGF IR. Ligand binding to IGF IR results in autophosphorylation of tyrosine residues in the β subunit. Docking molecules IRS-1 and Shc interact with phosphorylated tyrosines in the β subunit and activate the PI3K and MAPK pathways, respectively. Akt phosphorylation inhibits apoptosis and activates mammalian target of rapamycin (mTOR) leading to cell proliferation, growth and protein synthesis. PTEN negatively regulates Akt thus inhibiting its actions. Activation of Ras ultimately leads to phosphorylation of ERK, which stimulates cell proliferation. Binding of IGF II to the IGF IIR leads to degradation of IGF II via lysozyme mediated pathway.
archived. 10 years later in 1992, 520 individuals in this cohort were diagnosed with prostate cancer. Plasma IGF I and IGFBP 3 levels were measured in the 1982 samples. Men with high IGF I levels and low IGFBP 3 levels had increased risk for advanced-stage prostate cancer compared with men with low levels of both. The association was stronger in older men (Chan et al., 2002). Another cohort study (n=52) using blood samples from cases with histologically confirmed prostate cancer in Sweden showed an age-adjusted odds ratio for development of prostate cancer of 1.56. It was shown that the association between IGF I levels and prostate cancer risk was higher in the younger age group (Mantzoros et al., 1997). Overall, these studies show an increased risk of developing prostate cancer with elevated IGF I levels.

As previously mentioned, IGFBPs regulate IGF I bioavailability. IGFBP 2 is increased in malignant prostate tissue and IGFBP 3 is decreased, relative to normal epithelium (Tennant et al., 1996b). PSA is elevated in prostate cancer and cleaves IGFBP 3 (Rehault et al., 2001). IGFBP 3 inhibits IGF I interaction with IGF IR, therefore cleavage of IGFBP 3 by PSA reverses this inhibition. IGFBP 2, IGFBP 3 and IGFBP 5 expression changes with Gleason score. High levels of IGFBP 2, IGFBP 5 and low levels of IGFBP 3 are observed in high Gleason score tumours relative to low Gleason score or benign tissue (Figueroa et al., 1998). Increased IGFBP 2 may play an important role in positive growth regulation in prostate cancer. As well as elevated IGFBP 2 and decreased IGFBP 3 in prostate cancer tissue, serum IGFBP 2 is increased in prostate cancer relative to controls and is highly correlated with a rise in PSA (r =0.619) (Cohen et al., 1993). Serum IGFBP 3 levels are decreased in prostate cancer relative to controls (Kanety et al., 1993). As PSA levels rise this allows for liberation of more IGF I suggesting that IGF I plays an active role in prostate cancer progression. IGFBP 5 may enhance IGF I interaction with IGF IR as IGFBP 5 binds the extracellular matrix (Jones et al., 1993).

Signal transduction pathways downstream of the IGF IR also become de-regulated in cancer making the IGF pathway a target for inhibition in cancers overexpressing the IGF IR. PTEN, a tumour suppressor, is frequently lost or mutated in prostate cancer (Li et al., 1997). Loss of PTEN correlates with high Gleason score and advanced stage prostate cancer (Mc Menamin et al., 1999). PTEN negatively regulates Akt, therefore loss of PTEN leads to increased activity of Akt (Graff et al., 2000). Phospho Akt is a strong predictor of biochemical recurrence (Ayala et al., 2004) and is higher in high Gleason score (8 - 10) prostate cancer relative to PIN or lower Gleason scores (Malik et al., 2002).

1.6.3 Targeting the IGF signalling pathway
Due to the involvement of the IGF signalling pathway in cancer development and progression, numerous strategies have been developed to target this pathway, which
include reducing IGF I production, neutralising IGFs, anti-sense strategies, monoclonal antibodies (mAbs) against the IGF IR and IGF IR tyrosine kinase inhibitors.

1.6.3.1 Therapies targeting the IGF I ligand

As IGF I levels are associated with increased risk of prostate and breast cancer (Scherhhammer et al., 2005, Stattin et al., 2000), reducing circulating IGF I levels could reduce the incidence of cancer. Growth hormone-releasing-hormone (GHRH) antagonists have been developed. Growth hormone (GH) regulates synthesis and secretion of IGF I within the liver (Mathews et al., 1986). Therefore, GHRH antagonists inhibit GH release thus inhibiting IGF I synthesis and secretion. The peptide JV-1-38 is a GHRH antagonist that inhibited growth of NCI-M838 non-small cell lung cancer cells in vitro and in athymic mice (Szereday et al., 2003). JV-1-38 also exerted anti-tumour activity in prostate cancer models. Nude mice bearing subcutaneous tumours of LNCaP (androgen dependent), MDA-PCa-2b (androgen dependent) or DU145 (androgen independent) cells were treated with JV-1-38. Androgen sensitive models showed JV-1-38 potentiated anti-tumour effect following androgen deprivation by castration only relative to castrated controls. JV-1-38 alone inhibited the growth of androgen independent tumours (Letch et al., 2003). Pegvisomant, pegylated GH, is a GH receptor antagonist that was developed and approved for treatment of acromegaly where GH excess is seen. Pegvisomant had anti-tumour activity in 15 primary cultures of human meningiomas tumours implanted subcutaneously in nude mice (Mc Cutcheon et al., 2001).

An additional strategy targeting the IGF pathway is to neutralise IGF I/IGF II therefore inhibiting binding to the IGF IR. The inhibitory effects of IGFBPs on IGFs and the IGF-independent effects of IGFBPs have been investigated as possible therapeutics in cancer. In prostate cancer, epidemiological studies have demonstrated that high plasma levels of IGF I and low levels of IGFBP 3 are associated with an increased risk of prostate cancer development (Winter et al., 2001). IGFBP 3 is the most studied of the binding proteins as the majority of IGF I (70 %) in circulation is bound to IGFBP 3 as a complex with ALS (Baxter et al., 1989). Recombinant IGFBP 3 (rIGFBP 3) inhibits the growth of HER2-overexpressing breast tumours and potentiates Herceptin™ (Roche) activity in vitro and in vivo. MCF-7/HER 2-18 (HER 2 transfected cells) xenografts (female athymic mice) were treated with Herceptin™, rIGFBP 3 or both for 3 weeks. Herceptin™ treatment did inhibit tumour growth. However, when Herceptin™ and rIGFBP 3 were given in combination, a statistically significant reduction in tumour volume (76-106 %) was noted relative to controls (Jerome et al., 2006). M12 (malignant prostate cancer cell line) cells transfected with IGFBP 3 implanted subcutaneously into male nude athymic mice resulted in smaller tumours relative to controls. (Devi et al., 2002). Lung cancer (M-3LL) cells were used in vivo (female
nude mice) to test the effect of rhIGFBP 3 alone or with carboplatin (Bristol-Myers Squibb). In the lung cancer model, rhIGFBP 3 had a greater growth inhibitory effect than carboplatin. The combination of rhIGFBP 3 and carboplatin did not result in an enhancement of the antiproliferative effects of either agent alone (Alami et al., 2008). IGFBP 3 binds specific proteins on the cell surface such as the IGFBP-3R, which mediate inhibition of cell proliferation (Ingermann et al., 2010, Oh et al., 1993a). A mutant IGFBP 3 unable to bind IGF I and IGF II stimulated apoptosis in androgen independent PC-3 prostate cancer cells in vitro (Hong et al., 2002). Ingermann et al (2010) identified a novel cell death receptor mediating IGFBP 3 induced apoptosis. This study discovered that the IGFBP-3R binds specifically to IGFBP 3. In breast and prostate cancer tissue, IGFBP 3 and IGFBP-3R levels were significantly reduced relative to benign breast or prostate tissue, respectively. In subcutaneous models of breast (MDA-231 cells) and prostate (M12 cells) cancer, intratumour injection of IGFBP-3R resulted in a 40% reduction in tumour mass with little toxicity to the mice relative to controls (Ingermann et al., 2010). IGFBP 3 has been used in combination with other drugs. The retinoid X receptor-α (RXR) has been identified as a nuclear binding partner to IGFBP 3 which is involved in IGFBP 3 induced apoptosis (Liu et al., 2005). A RXR ligand, VTP194204 (Vitae Pharmaceuticals) and rhIGFBP 3 in combination were shown to have a synergistic effect on prostate cancer cell apoptosis in vitro and in vivo in a LAPC-4 (androgen dependent) subcutaneous tumour model. In vivo, treatment with both VTP194204 and rhIGFBP 3 inhibited tumour growth and reduced serum PSA levels relative to either treatment alone (Liu et al., 2005). Prostates of IGFBP 3 knockout mice (IGFBP 3 KO) failed to undergo apoptosis or develop prostate tumours. Following castration, lung metastases developed in 55% of IGFBP 3 KO mice relative to controls. The IGFBP 3 KO was crossed with a c-Myc driven prostate cancer model, which develops slow growing, non-metastatic tumours. Cell lines derived from the Myc:IGFBP 3 KO tumours were more aggressive in terms of proliferation, invasion and colony formation relative to Myc tumour cell lines in vitro (Mehta et al., 2011). However, human ectocervical cells, immortalised by retroviral transduction with the human papillomavirus type 16 E6/E7 secreted high levels of IGFBP 3 and exerted an increased sensitivity to IGF I, including increased cell proliferation (Baege et al., 2004).

IGFBP 2 is the second most abundant IGFBP in circulation and it is often elevated in prostate cancer and involved in cancer progression (Busund et al., 2005, Degraff et al., 2009). Therapeutic strategies have been developed to block IGFBP 2 activity. In androgen dependent LAPC-4 prostate cancer cells, IGFBP 2 stimulated growth, but had a growth inhibitory effect on normal prostatic epithelial cells (Moore et al., 2003). MDA-MB-231BP-2 (oestrogen receptor negative) breast cancer cells constitutively overexpressing IGFBP 2 grew quicker and were more resistant to paclitaxel in vitro and in vivo relative to the parental
cells (So et al., 2008). By using an antisense oligonucleotide to IGFBP 2 (OGX-225) the aggressive phenotype of MDA-MB-231BP-2 cells was decreased in vitro and in vivo (So et al., 2008). A Phase I clinical trial has been established in order to test the side effects of using a DNA plasmid based vaccine encoding amino acids 1-163 of IGFBP 2 in advanced ovarian cancer (www.clinicaltrials.gov, 2011).

IGFBP 1 is the least studied of the IGFBPs and little is known about its role as a possible therapy in cancer. IGFBP 1 has been reported to inhibit motility of the bone-seeking metastatic breast cancer cell line, MDA-231BO in vitro by inhibiting the interaction of the integrin α5β1 with the IGF IR (Zhang and Yee, 2002).

IGFBP 5 overexpression can accelerate progression to androgen independence in a human prostate LNCaP tumour model. After castration tumour growth and serum PSA levels increased faster in mice bearing IGFBP 5-transfected LNCaP tumours relative to controls (Miyake et al., 2000). However, in human breast cancer cells (MDA-MB-231 and Hs578T), IGFBP 5 inhibits growth in vitro and in vivo in nude mice (Butt et al., 2003). IGFBP 5 can act as an inhibitor to tumour growth by inhibiting angiogenesis. Overexpression of IGFBP 5 in HUVECs (human umbilical vein endothelial cells) treated with VEGF led to inhibition of tube formation and cell proliferation. In vivo, following injection of rhIGFBP 5, SKOV3 ovarian cancer cells implanted subcutaneously in nude mice showed a 70 % reduction in tumour volume relative to controls (Rho et al., 2008). Due to conflicting data, IGFBP 5 needs to be investigated further before being considered as a cancer therapeutic.

IGFBP 6 has a high affinity for IGF II (Chandrashekaran et al., 2007). IGFBP 6 inhibits PC-3 (androgen independent) and LNCaP (androgen dependent) prostate cancer cell proliferation following treatment with diethylstilbestrol (DES), a synthetic oestrogen used as an androgen antagonist in the treatment of advanced human prostate cancer (Koike et al., 2005). Upon treatment of PC-3 cells with DES, an increase in IGFBP 6 was noted. rhIGFBP 6 alone inhibited cell proliferation suggesting that IGFBP 6 may be involved in the effect of DES in androgen independent human prostate cancer (Koike et al., 2005). IGFBP 6 induced programmed cell death in non-small cell lung cancer (NSCLC) cells transduced with IGFBP 6 in vitro. Intratumoural injection of an adenovirus expressing IGFBP 6 under the control of a cytomegalovirus (CMV) promoter (Ad5CMV-BP6) in NSCLC subcutaneous tumours established in nude mice reduced the size of NSCLC xenografts by 45% relative to controls (Sueoka et al., 2000). In a neuroblastoma model, IGFBP 6 inhibits cell proliferation in SK-N-SH cells in vitro and subcutaneous tumour development in nude mice in vivo, further supporting IGFBP 6 as a candidate for cancer therapy (Seurin et al., 2002). As IGFBP 6 binds IGF II with higher affinity compared with IGF I, IGFBP 6 may be useful in cancers where IGF II is primarily involved.
The body naturally produces IGFBPs, therefore they may not stimulate an immune response, making them tolerable to patients. IGFBPs can also have IGF dependent and independent effects; under certain conditions the IGFBPs can enhance IGF activity thus leading to an increase in proliferation and tumour growth (Baege et al., 2004, Miyake et al., 2000). All of the IGFBPs are regulated by proteases. Such proteases include PAPP-A and PSA, which cleave IGFBP 3 and 4 (Rehault et al., 2001, Boldt and Conover, 2007). Recently it has been shown that overexpression of PAPP-A in ovarian cancer cells (SKOV3) led to accelerated anchorage-independent growth in soft agar relative to a SKOV3 cells expressing a mutant PAPP-A with reduced proteolytic activity. In vivo, accelerated growth of PAPP-A-overexpressing SKOV3 cells was seen relative to mutant PAPP-A or controls (Boldt and Conover, 2011). Therefore, IGFBPs may not have a long half-life in circulation due to cleavage by such proteases. IGFBP 4 is inhibitory to IGFs without any known IGF independent actions. Therefore, a protease resistant IGFBP 4 would be useful to overcome proteolytic cleavage of IGFBPs.

A protease resistant form of IGFBP 4 was used in a porcine model of neointimal hyperplasia. Upon infusion of the protease resistant IGFBP 4 into the arteries of hypercholesterolemic pigs, a significant decrease in lesion formation was observed in the protease resistant IGFBP 4 treatment group relative to controls or pigs treated with wildtype IGFBP 4. A decrease of 53.3 ± 6.1% in lesion development within blood vessels was seen in hypercholesterolemic pigs treated with protease resistant IGFBP 4 compared with controls or wildtype IGFBP 4. The decrease in neointimal expansion was due to decreased cell proliferation (Nichols et al., 2007). Overexpression of wildtype IGFBP 4 delayed the onset of subcutaneous prostate tumour formation in athymic nude mice in vivo using M12 prostate cancer cells transfected with IGFBP 4. In vitro, overexpressed IGFBP 4 increased apoptosis and decreased cell proliferation in the M12 transfected cells relative to controls, while in vivo M12 cells transfected with IGFBP 4 grew significantly slower than controls (Damon et al., 1998). In a subcutaneous nude mouse model of colon cancer, peritumoural injection of a mammalian vector (pcDNA3) containing wildtype IGFBP 4 cDNA increased apoptosis and decreased mitosis of HT-29 colon cancer cells (Durai et al., 2007). In the Harmey group, a protease resistant IGFBP 4 was previously developed. In vivo, following injection of 4T1.2 cells transfected with the protease resistant rat IGFBP 4 (dB4) grew significantly slower than controls or cells expressing wildtype IGFBP 4. Inhibition of tumour growth by dB4 was accompanied by increased endothelial cell apoptosis and mice bearing dB4-expressing tumours took longer to reach a mean tumour diameter (MTD) of 17 mm relative to controls or wildtype IGFBP 4 tumours (Ryan et al., 2009). Therefore, dB4 may be a strong candidate as a cancer therapeutic.
A second strategy to neutralise IGFs is a soluble receptor. Dunn et al (1998) created a soluble mutant IGF IR, which functioned as a decoy receptor. It was shown to inhibit cell motility and adhesion *in vitro* in MDA-MB-435 breast cancer cells transfected with the dominant negative form of the IGF IR. When MDA-MB-435 cells expressing the dominant negative form of the IGF IR were injected into the mammary fat pad of female nude mice, metastasis to the lungs, liver, lymph nodes and lymph vessels was significantly decreased compared with vector controls (Dunn et al., 1998).

Antibodies to IGF I and IGF II have also been described. KM1468, a rat monoclonal antibody neutralised IGF I and IGF II and inhibited development of bone metastases in MDA-PCa-2b prostate cancer cells injected into subcutaneously implanted human bone fragments (Goya et al., 2004).

**1.6.3.2 Anti-sense oligonucleotides and RNAi to inhibit IGF IR expression**

Overexpressing the IGF IR leads to transformation of cells which is important in cancer cell development (Kaleko et al., 1990). Therefore, decreased expression of the IGF IR may inhibit IGF IR transforming ability along with its cell proliferative and anti-apoptotic effects. Antisense and ribonucleic acid (RNA) interference are two methods, to decrease IGF IR expression. Antisense RNA involves the introduction of antisense RNA molecules or oligonucleotides into the cell. Binding of the RNA molecules or oligonucleotides to a complementary sequence in the target mRNA inhibits translation of mRNA or causes degradation by RNase H (Walder and Waider, 1988). Intratumour injection of antisense oligonucleotides to IGF IR inhibits the growth of C4HD mammary adenocarcinomas *in vivo* (Salatino et al., 2004). Treatment of the breast cancer cell line MDA-MB-435 with antisense IGF IR showed a decrease in cell proliferation *in vitro* and when cells were injected subcutaneously into SCID mice an increase in survival was reported relative to controls (Chernicky C.L. et al., 2000). An antisense oligonucleotide to the IGF IR was tested in a clinical trial for malignant astrocytomas. *Ex vivo*, IGF IR oligonucleotide treatment of autologous glioma cells induced apoptosis. *In vivo*, a host response was seen without unusual side effects (Andrews et al., 2001). The efficacy of systemic administration of antisense oligonucleotides is restricted by limited cellular uptake and non-specific toxicity due to poor target sequence specificity.

RNA interference (RNAi) is a second method to decrease IGF IR expression. RNAi is a process by which long double stranded RNAs are cleaved into short nucleotide fragments. The cleavage products, short interfering RNAs (siRNAs) are incorporated into a RNA inducing silencing complex where the siRNA acts as a guide to target the cleavage of complementary mRNA (Fire et al., 1998). siRNAs have been used to decrease IGF IR levels and signalling in cancer. *In vitro*, silencing of the IGF IR in LNCaP, PC-3 and DU145
prostate cancer cells enhanced their sensitivity to DNA damaging agents (mitoxantrone, etoposide, nitrogen mustard and ionizing radiation) (Rochester et al., 2004). However, the delivery of these agents has similar problems as that of the antisense oligonucleotides.

1.6.3.3 Monoclonal antibodies to inhibit IGF I and the IGF IR
Therapies targeted at inhibiting IGF IR activation are furthest along in terms of clinical development. The two main IGF IR inhibiting strategies are monoclonal antibodies (mAb) and small molecule tyrosine kinase inhibitors.

Neutralising antibodies to block receptor-ligand interactions leads to receptor internalisation and degradation (Burtrum et al., 2003). Down-regulation of the IGF IR is the most important mechanism of action of these drugs. The first available mAb blocking the IGF IR was αIR3, a mouse monoclonal IgG1 antibody. αIR3 inhibited growth of MCF-7 breast cancer cells in vitro, but did not inhibit in vivo growth (Arteaga et al., 1989). However, αIR3 inhibited subcutaneous growth of other types of cancers including MDA-MB-231 breast cancer cells (Arteaga et al., 1989) and rhabdomyosarcoma (Kalebic et al., 1994). However, αIR3 can act as an IGF I agonist stimulating phosphorylation of the IGF IR (Kato et al., 1993). Therefore, this antibody was not developed clinically, but it led to development of fully humanised mAbs.

CP-751,871 (Figitumumab, Pfizer), a fully human IgG2 antibody with high affinity for the human IGF IR, has the largest clinical test to date compared with other IGF IR mAbs. CP-751,871 induced down-regulation of the IGF IR protein in NIH 3T3/IGF-IR (IGF IR overexpressed), MCF-7 breast cancer cells and RPMI 8226 multiple myeloma cells in vitro and showed significant anti-tumour activity in athymic mice bearing subcutaneous NIH3T3/IGF-1R tumours relative to controls (Cohen et al., 2005). A phase I pharmacokinetic and pharmacodynamic study in patients with multiple myeloma showed no dose limiting toxicities when CP-751,871 was administered (Lacy et al., 2008). Administration led to a decrease in serum IGF I at high doses, thus suggesting systemic IGF IR inhibition. Phase I dose escalation studies were also conducted in patients with refractory solid tumours. Some of the most common adverse effects were hyperglycaemia, anorexia, nausea, diarrhoea and fatigue. At a dose of 20 mg/kg, 10 out of 15 patients experienced disease stabilisation (Lacy et al., 2008). Treatment with CP-751,871 increased serum insulin and GH levels and caused a modest increase in blood glucose, suggesting target inhibition (Haluska et al., 2007). In a phase II study, CP-751,871 combined with paclitaxel and carboplatin was shown to be safe and effective in patients with treatment-naive, locally advanced, or metastatic lung cancer (Karp et al., 2009).

IMC-A12 (Cixutumumab, ImClone Systems) is a fully human IgG1 antibody. IMC-A12 inhibited subcutaneous growth of androgen dependent (LuCaP 35) and androgen
independent (LuCaP 35V) prostate cancer cell lines and induced apoptosis and growth cycle arrest (Wu et al., 2005). In MCF-7 breast cancer cells, IMC-A12 administration led to inhibition of both MAPK and Akt signalling and demonstrated strong anti-tumour activity in subcutaneous tumour models (Burtrum et al., 2003). IMC-A12 works effectively alone and in combination with the epidermal growth factor receptor (EGFR) blocking antibody cetuximab (C225, Merck) in head and neck cancer cell lines (183A and TU159) and tumour xenografts (Barnes et al., 2007). IMC-A12 has been tested in phase I trials in adults and children with advanced solid tumours. Toxicities included hyperglycaemia, anaemia, psoriasis and infusion related reaction (Higano et al., 2007).

AMG-479 (Amgen) is a fully human IgG1 antibody with high affinity for the human IGF IR and no cross reactivity with the insulin receptor. AMG-479 reduced cell viability in the pancreatic cell lines BxPC-3 and MIAPaCa2. Its mechanism of action was different in the two cell lines, being pro-apoptotic in BxPC-3 and anti-mitogenic in MIAPaCa2 cells. AMG-479 inhibited subcutaneous tumour growth of BxPC-3 and MIAPaCa2 pancreatic carcinoma xenografts relative to controls (Beltran et al., 2009). AMG-479 in combination with cetuximab in cetuximab-refractory head and neck squamous cell carcinoma showed a decrease in phosphorylated IGF IR (Chung et al., 2010). Phase I trials performed with patients with solid tumours and non-Hodgkin’s lymphoma, revealed no severe toxicity of this antibody (Tolcher et al., 2009). Clinical trials are now underway to determine the anti-tumour activity of AMG-479 in numerous cancers such as breast, colorectal, lung and pancreatic cancer (www.clinicaltrials.gov (2011)).

R1507 (Roche), a recombinant human IgG1 monoclonal IGF IR antibody, was first tested in NSCLC cell lines, showing that high levels of total IGF IR and not phosphorylated IGF IR are associated with relative sensitivity to the R1507 when administered as a single agent. However, its effect was enhanced when used in combination with the EGFR small molecule inhibitor Erlotinib (Roche) in R1507-sensitive NSCLC cells in vitro (Gong et al., 2009). R1507 was tested in 6 osteosarcoma cell lines (OS1, OS2, OS9, OS17, OS31 and OS33) implanted subcutaneously in SCID mice. R1507 was administered either as a single agent or in combination with the mTOR inhibitor, rapamycin. R1507 alone delayed tumour growth in four of six osteosarcoma xenografts and rapamycin alone enhanced Akt phosphorylation in all the tumours. Phosphorylation of Akt was inhibited when rapamycin was administered in combination with R1507 (Kolb et al., 2010). The safety and pharmacokinetics of R1507 was evaluated in patients in a phase I trial with solid tumours and lymphomas. Hyperglycaemia and lymphopenia were common side effects and a significant reduction in IGF IR expression on peripheral blood mononuclear cells was observed (Rodon et al., 2007). A phase II trial was undertaken in patients with recurrent or refractory sarcomas (Patel et al., 2009) and while initial activity was observed subsequent
findings following a portfolio review led to termination of the clinical development of this agent.

SCH-717454 (Robatumumab, Schering-Plough), a fully humanised monoclonal antibody previously known as 19D12, was tested *in vitro* and *in vivo* under the Paediatric Preclinical Testing Program. *In vitro* tests of this antibody on 23 cell lines including neuroblastoma, osteosarcoma, Ewing's sarcoma, kidney/rhabdoid and gliomas showed little effect on cell growth, whereas *in vivo* it possessed broad anti-tumour activity in 43 xenograft models, the strongest effects seen in neuroblastoma, osteosarcoma and Ewing's sarcoma models (Kolb *et al.*, 2008). SCH-717454 was further evaluated in rhabdomyosarcoma, neuroblastoma and osteosarcoma tumour xenografts. It was effective as a single agent with enhanced activity in combination with cisplatin or cyclophosphamide. *In vivo* results showed tumour growth inhibition by SCH-717454 was due to a reduction in cell proliferation and angiogenesis (Wang *et al.*, 2010). When SCH-717454 was initially tested in healthy volunteers, no adverse side effects were seen 8 weeks post dosing (Seraj *et al.*, 2007). SCH-717454 is currently being evaluated in a Phase II trial in patients with relapsed or recurrent colorectal cancer and in patients with relapsing sarcomas (Ewing's sarcoma) (www.clinicaltrials.gov, (2011)).

Monoclonal antibody EM164 (Immunogen) inhibited proliferation of several human cancer cell lines *in vitro*, including breast, lung, colon, cervical, ovarian, pancreatic, melanoma, prostate, neuroblastoma, rhabdomyosarcoma and osteosarcoma lines. Tumour regression was also seen in established BxPC-3 human pancreatic subcutaneous tumours treated with EM164 relative to untreated controls with increased anti-tumour efficacy in combination with the cytotoxic agent, gemcitabine (Eli Lilly and Co.) (Maloney *et al.*, 2003).

Treatment of MCF-7 breast cancer cells with AVE-1642, the humanized version of EM164 conjugated with quantum dots led to down-regulation of IGF IR levels and reduction of cell proliferation *in vitro* (Zhang *et al.*, 2009). Intravenous (IV) injection of AVE-1642 conjugates (with quantum dots or small-molecule fluorophores) to mice bearing subcutaneous tumours of human-IGF IR expressing mouse embryo fibroblasts or MCF-7 breast cancer cells, specifically targeted the tumour xenograft following *in vivo* fluorescent imaging and were therefore suitable to detect the expression and down-regulation of IGF1R *in vivo* (Zhang *et al.*, 2009). AVE-1642 has also demonstrated activity in chemoresistance towards 5-Fluorouracil (5-FU) and oxaliplatin (Sanofi-Aventis) for chemoresistant metastatic colorectal cancer (HT-29 cells) *in vitro* (Dallas *et al.*, 2009). A dose escalation study using AVE-1642 was conducted in multiple myeloma patients and was well tolerated (Moreau *et al.*, 2007).

MK-0646, a humanised IgG1 monoclonal antibody (Pierre Fabre), inhibited subcutaneous tumour growth in nude mice bearing either human breast cancer cells (MCF-7) or NSCLC cells (A549). Further inhibition of A549 tumour growth was observed in mice
treated with the anti-IGF IR antibody combined with vinorelbine (Pierre-Fabre) or an anti-EGFR antibody, C225 (Goetsch et al., 2005). MK-0646 also exhibited potent inhibition of subcutaneous tumour growth in MDA-MB-231 breast cancer mouse models (Pandini et al., 2007). MK-0646 is currently in phase II clinical trial in combination with irinotecan (Pfizer) and/or cetuximab in colorectal cancer (Watkins et al., 2009).

Recently Biogen have developed a range of monoclonal and bispecific antibodies against the IGF IR. Combination of two inhibitory IGF IR antibodies with distinct epitopes for the receptor (BIIB4 and BIIB5) were more effective in osteosarcoma (SJSA-1 cells) and hepatocellular carcinoma (HepG2 cells) subcutaneous tumour models compared with the use of the monospecific antibodies (Dong et al., 2010).

1.6.3.4 Tyrosine kinase inhibitors to block IGF IR activation

A second group of inhibitors targeting IGF IR activation are small molecule tyrosine kinase inhibitors (TKIs). TKIs inhibit tyrosine kinase activity by binding the adenine triphosphate (ATP) binding site or substrate binding site in the kinase domain of the IGF IR. TKIs have a higher affinity for IGF IR than the IR, thus minimising cross-reactivity with the IR. One advantage of TKIs over mAbs is the ease of oral administration. A number of TKIs are currently in clinical development.

Some of the earliest TKIs were tyrphostins such as AG1024 (Merck), a synthetic protein kinase inhibitor which was not specific to the IGF IR as it cross-reacted with the IR (Parrizas et al., 1997a). This led to the production of NVP-AEW541 (Novartis), a novel tyrosine kinase inhibitor to the IGF IR. NVP-AEW541 is 27-fold more selective for IGF IR than the IR (Garcia-Echeverria et al., 2004). NVP-AEW541 has anti-proliferative effects in vitro and in vivo in neuroblastoma (HTLA-230 or SK-NBE2c cells). In vitro, NVP-AEW541 also increased apoptosis relative to controls, while in vivo 100 mg/kg/day significantly inhibited the growth of subcutaneous neuroblastoma tumours in nude mice relative to controls without any major toxic effects (Tanno et al., 2006). In an SKBR3/Tr (breast cancer cells resistant to trastuzumab (Roche)) model of acquired trastuzumab resistance, NVP-AEW541 inhibited growth of the resistant cells, to a comparable level to that of the trastuzumab sensitive parental cells when given in combination with trastuzumab. Therefore, NVP-AEW541 improved the response to trastuzumab in HER2-positive breast cancer cells (Browne et al., 2011). Numerous studies have examined toxicity of NVP-AEW541 due to high homology between the tyrosine kinase domains of IGF IR and IR (Ullrich et al., 1986). In a mouse model of Ewing’s sarcoma, no increase in serum glucose was seen. In contrast, there was a significant decrease in serum glucose due to increased glucose uptake at a cellular level. However, this was only evident in cells expressing IGF IR and IR and was not observed when only IR was present (Manara et al., 2007). NVP-
AEW541 given at a dose of 50mg/kg twice daily in an orthotopic pancreatic cancer (BxPC-3 cells) model led to an increase in blood glucose levels and a 16% decrease in mouse body weight (Moser et al., 2008), suggesting that the IR could be affected by NVP-AEW541 (Garcia-Echeverria et al., 2004). This inhibition may be due to dosing regime and the dose given. NVP-AEW541 was only tested pre-clinically and was not tested in clinical trials.

INSM-18 (Insmed), also known as nordihydroguaiaretic acid (NDGA), is a small molecule inhibitor of the IGF IR, which also targets the HER 2 receptor. MCF-7 breast cancer cells treated with low doses of INSM-18 showed inhibition of IGF IR phosphorylation and inhibition of phosphorylated Akt. In mouse mammary carcinoma MCNeuA cells (overexpress the HER2/neu receptor) derived from a spontaneous tumour in a female neu transgenic mouse, INSM-18 inhibited ligand independent phosphorylation of HER2/neu and IGF I-induced growth. INSM-18 treatment also decreased the activity of the IGF IR and the HER2/neu receptor in vivo in MCNeuA subcutaneous tumours (Youngren et al., 2005). INSM-18 has demonstrated anti-proliferative activity in LNCaP prostate cancer cells in vitro (Ryan et al., 2006). Two single dose phase I trials conducted in healthy volunteers showed INSM-18 was well tolerated. A dose escalation phase I/II trial has been completed in 15 patients with relapsed prostate cancer where INSM-18 was well tolerated up to the maximum dose (Ryan et al., 2008).

OSI-906 (OSI Pharmaceuticals) is a potent small molecule inhibitor of the IGF IR. In a pre-clinical mouse model of lung cancer (NCI-H292 cells), 18FDG-PET was used to monitor the effect of OSI-906 on subcutaneous tumour growth. A decrease in glucose uptake within the tumour was observed following OSI-906 administration. The decrease in glucose uptake coincided with a decrease in phosphorylated IGF IR and reduced PI3K and MAPK activity, showing tumour arrest in lung tumours relative to controls (McKinley et al., 2011). Phase I trials of continuous and intermittent administration of OSI-906 in patients with advanced solid tumours have only reported mild hyperglycaemia (Lindsay et al., 2009).

XL228 (Exelixis) is a small molecule TKI targeting the IGF IR, FGFR 1-3 and SRC family kinases. Phase I studies tested XL228 in patients with solid tumours and hematological malignancies. Side effects included hyperglycaemia, nausea, fatigue, anorexia and flushing (Smith et al., 2008).

The IGF system is emerging as a promising new target in cancer treatment. Early clinical testing has shown that IGF IR inhibition is well tolerated, with hyperglycaemia as the main side effect. Single agent activity of these agents is well documented and combination studies with other conventional therapies such as chemotherapy, hormone therapy and radiotherapy are currently being explored. Identification of biomarkers may help select patient populations that will benefit from IGF pathway directed therapies. However, newer
therapies are needed which avoid the hyperglycaemic effects seen with many existing IGF
IR-targeted agents.

1.7 Animal models of prostate cancer
The selection of suitable animal models in which to assess novel therapies is important pre-
clinically. It is crucial that an appropriate model is chosen to establish the potential of such
therapies. Elucidation of the molecular mechanisms underlying prostate cancer initiation and
progression has been facilitated by laboratory and clinical models of the disease. Due to the
heterogeneity of prostate cancer between and within patients, numerous complementary
model systems have emerged including dog, rat, transgenic mice and xenografts. Aside
from man, dog is the only animal known to spontaneously develop high grade PIN and
prostate adenocarcinomas (Waters et al., 1998).

Canine disease shares numerous similarities with human disease, including
morphologic and phenotypic heterogeneity, metastatic propensity and development of
androgen independence and late age of onset. These similarities make canines an attractive
model, but this model has limitations due to low incidence of spontaneous disease and the
cost of maintaining dog colonies.

Several rat strains develop prostate adenocarcinomas after a long latency period.
Prostate cancer can also be induced in rats by stimulation with androgens, oestrogens or
treatment with chemical carcinogens (Cavalieri et al., 2002), but prostate tumours formed in
this way display variations in reproducibility and reliability. The most beneficial resource
developed from spontaneous and induced rat tumours is a set of metastatic and non­
metastatic cell lines developed from the Dunning R3327 rat (Isaacs et al., 1986). These
sublines have been utilised in studies on androgen responsiveness and metastatic
progression (Bussemakers et al., 1992, Quarmby et al., 1990).

Transgenic mice have been used in modelling numerous cancer types. In prostate
cancer, these models have not fared well as they are not representative of the human
disease. The murine prostate does not spontaneously develop neoplasia and the anatomy
of the murine prostate differs greatly from that of the human prostate. The murine prostate
consists of three lobes, dorsolateral, ventral and anterior, which are positioned around the
urethra. The human prostate is alobular, but can be divided into three zones- central,
peripheral and transitional and it envelops the upper part of the urethra.

Despite these differences, prostate cancer has been engineered in mouse strains
using prostate specific promoters to express oncogenes. Generation of transgenic mice
overexpressing numerous oncogenes, growth factors and kinases have been developed.
The most commonly used transforming agent is the gene for SV40 T antigens. SV40
oncoproteins inactivate the tumour suppressor p53 which is involved in tumour suppression
This pathway is also mutated in human prostate cancer (Bookstein et al., 1993). The SV40 antigens and several other oncoproteins have been utilised in the murine prostate along with a variety of promoters.

The rat minimal probasin promoter belongs to a family of ligand carrier proteins localised to epithelial cell nuclei and ducts of dorsolateral and ventral prostate (Greenberg et al., 1994). The minimal probasin promoter is subject to steroid hormone regulation and developmental regulation (Greenberg et al., 1994). The minimal probasin promoter was used to develop the transgenic adenocarcinoma mouse prostate (TRAMP) model of prostate cancer. The TRAMP model contains the minimal probasin promoter that drives expression of the SV40 large T antigen. TRAMP mice develop high grade PIN or prostate cancer 12 weeks after birth and develop metastases after 30 weeks with the majority of metastases forming in the lung and lymph nodes (Gingrich et al., 1997, Greenberg et al., 1995). The TRAMP model has been used to analyse changes in the course of androgen deprivation following castration (Gingrich et al., 1997), to assess aberrant growth factor signalling in prostate cancer (Kaplan et al., 1999) and to test chemotherapeutics (Gupta et al., 2004).

The promoter of rat C3 (1) is an androgen regulated promoter that promotes expression of a ventral lobe protein using SV40 large T antigen gene. These mice develop low grade PIN to high grade PIN to invasive carcinoma, but their use is limited due to multiple tissue sites of tumour onset (Maroulakou et al., 1994). However, this model has been valuable in the study of progression from low grade PIN to invasive carcinoma, thus supporting PIN as a precursor to carcinoma. Transgenic mice overexpressing IGF I under the control of the keratin 5 promoter in basal cells in various tissues develop PIN and carcinoma by nine months of age (Digiovanni et al., 2000). However, the existing transgenic models have limitations. The SV40 based models do not allow analysis of the initiating events as SV40 viral oncoprotein does not cause prostate cancer in humans. In addition, the majority of transgenic models do not reliably metastasise to bone, a common feature in prostate cancer.

In human xenograft models of prostate cancer, xenografts are created by implantation of human tissue or cells into immunodeficient rodents such as nude, SCID (severe combined immunodeficiency) or NOD (non-obese diabetic) SCID mice. Xenografts allow investigation of human prostate cancer and facilitate determination of the influence of microenvironment on growth and behaviour of tumour cells. Xenograft models are commonly used to test therapeutic agents for clinical trials serving as a link between laboratory and clinic. Subcutaneous implantation of human cancer tissue or cells has traditionally been utilised to test potential therapeutics. This model is easy to use, is technically straightforward and creates a great amount of data, but it is not relevant to
clinical cancer. One of the obvious drawbacks is that subcutaneous tumours do not reproduce the microenvironment of the prostate and rarely metastasise.

To overcome these issues orthotopic models using implantation of tissue or cells into the corresponding anatomical site have been established. Metastasis formation is more efficient and mimics that of human metastasis when cells or tissue are implanted orthotopically. In one model, PC-3 cells were transduced with green fluorescent protein (GFP) and implanted subcutaneously in order to select high expressing GFP-fluorescent tumours. Subcutaneous tumour fragments expressing high levels of GFP were then implanted into the prostate of nude mice. Subsequent metastasis formation was seen in bone (Yang et al., 1999). By implanting tissue or cells orthotopically, targeting factors involved in invasion can be carried out in a more clinically relevant site and testing novel therapeutics may better reflect activity in patients. Kuo et al (1993) used an in vivo SCID beige model of NSCLC and showed that cisplatin had significant effects against lung tumours growing orthotopically, but had no effect against the same tumours growing subcutaneously (Kuo et al., 1993). One disadvantage of orthotopic implantation is that for certain anatomical sites, including prostate, implantation requires surgical skills and is more time consuming than subcutaneous implantation of cells or tissue. Caliper measurement cannot be carried out; therefore determining endpoints for effects of therapy can be difficult. There have been many significant developments in orthotopic implantation techniques to study cancer and metastasis.

Many animal tumours do not give rise to spontaneous bone metastasis despite orthotopic implantation. Therefore, experimental bone metastasis models are used. Intracardiac injection of cell lines into the left ventricle of immunodeficient mice gives rise to bone metastasis in vivo (Chu et al., 2008). Advancements in small animal imaging now allows for non-invasive imaging of animals bearing orthotopic tumours and metastasis.

1.7.1 Non-invasive imaging of animal models of cancer

A variety of non-invasive imaging techniques have been optimised for small animal imaging, including magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single photon emission tomography (SPECT), fluorescent imaging and bioluminescent imaging (BLI). Non-invasive imaging allows monitoring of changes in blood perfusion or volume within the tumour and metastatic lesion, tracking tumour specific markers and monitoring effects of novel therapeutics (Genove et al., 2005, Lewin et al., 1999, Mc Kinley et al., 2011). MRI and CT rely on energy-tissue interactions whereas PET and BLI rely on injection of molecular probes to visualise tumours.

MRI works on the principle that unpaired nuclear spins (hydrogen bonds in water) align themselves in a magnetic field. A radio frequency is used to alter the alignment of
spins and the return to baseline is the change in electromagnetic flux. The rotating magnetic field is detected by a scanner and an image is constructed of the scanned body. High resolution is commonly used for screening transgenic mice for tumours as it can visualise tumour size, location and metastatic burden. MRI analysis can also be useful to quantify vascular volume and capillary flow of deep tumours (Lewin et al., 1999, Neeman et al., 2001). MRI has reportedly been used to detect endothelial cell proteins such as E-selectin by using a superparamagnetic contrast agent obtained by grafting a synthetic mimetic of sialyl Lewisx, a natural ligand of E-selectin expressed on leukocytes, on the dextran coating of ultrasmall particles of iron oxide (Boutry et al., 2006). Another study used an adenovirus to deliver an MRI reporter gene, which encoded metalloproteins from the ferritin family. When cells sequestered iron a superparamagnetic compound was formed, allowing cells to construct the MRI contrast agent in situ (Genove et al., 2005).

CT (X-ray CT) imaging measures x-rays as they pass through tissue. Due to differences in absorption between bone, fat, water and air, high contrast images of anatomical structures are obtained. Compared with MRI, CT has poor soft tissue contrast and does not distinguish between tumours and surrounding tissue. To overcome this, an iodinated contrast agent is injected, which delineates organs and tissues, as different tissue types will absorb the contrast agent at different rates. High resolution CT is frequently used in mice to detect lung tumours (De Clerck et al., 2004). MRI is usually preferred over CT for tumour screening, but CT is useful in certain circumstances such as bone metastasis (Mahner et al., 2008) as CT imaging is faster than MRI and gives a better image of bone.

PET imaging is a technique used to detect decaying nuclides such as $^{11}$C, $^{18}$F and $^{124}$I. Once administered the decaying nuclides emit positrons that collide with free electrons converting matter to energy in the form of $\gamma$ rays. $\gamma$ rays are detected by numerous detectors surrounding the subject. The radionuclides are typically conjugated to small molecules that can bind to receptors or other cellular targets. For example, $^{18}$F fluorodeoxyglucose is used to detect tumours due to increased glucose uptake and metabolism and has been used extensively in testing novel therapeutics such as the IGF IR TKI, OSI-906. Administration of OSI-906 in a subcutaneous model of lung cancer (NCI-H292 cells) led to a decrease in glucose uptake following treatment (McKinley et al., 2011) demonstrating that PET imaging may be useful in determining response to drug therapies. $^{18}$F labelled thymidine has also been used to measure proliferation in NSCLC tumours (Yap et al., 2006). However, PET imaging is a costly approach to monitoring mouse models of cancer and in addition to a PET scanner, a cyclotron and radiochemistry facility is required for daily use of this technology.

SPECT imaging is used in experimental oncology as it detects gamma radionuclides to track individual molecules or cells. Gamma emitting nuclides are inexpensive and readily
available. Molecules or cells are labelled with $\gamma$-ray emitting nuclides such as $^{123}$I and $^{125}$I then administered to animals and labelled molecules or cells can then be monitored by sodium iodide gamma cameras. SPECT has been used to image programmed cell death induced by anti-Fas antibody injection in BALB/c mice and cyclophosphamide treatment of transplanted 38C13 murine B cell lymphomas (Blankenberg et al., 1998). Wild type RG2 glioma or W256 mammary carcinoma cells were injected into one flank of rats and RG2TK+ glioma or W256TK+ mammary carcinoma cells that had been transduced in vitro with the Herpes simplex virus 1-tyrosine kinase gene were injected into the opposite flank. Herpes simplex virus 1-tyrosine kinase expression was visualised using $^{131}$I-labelled 2'-fluoro-2'-deoxy-1-β-d-arabinofuranosyl-5-ido-uracil. High levels of radioactivity (>1% dose) were localized to areas of Herpes simplex virus 1-tyrosine kinase gene expression. In contrast, significantly lower levels of radioactivity (<0.01%) were observed in the surrounding non-transduced tumour tissue or contralateral wild type tumours (Tjuvajev et al., 1996). SPECT has also been used to follow radiolabelled HER2-specific antibodies. Two anti-HER2/neu mAbs (TA1 and 520C9) were radioiodinated. Internalization and cellular processing of the labelled mAbs were assessed in SKOV3 9002-18 ovarian cancer cells in vitro (Zalutsky et al., 1999). Another study synthesised and evaluated the in vivo biodistribution of 2 radiolabelled urea derivatives with high affinity for prostate specific membrane antigen (PSMA), an integral membrane glycoprotein. SCID mice harbouring MCF-7 (breast, PSMA-negative), PC-3 (prostate, PSMA-negative) or LNCaP (prostate, PSMA-positive) subcutaneous tumours showed that imaging PSMA-positive prostate cancer was achieved using low molecular weight agents (Foss et al., 2005).

Fluorescent imaging is an inexpensive tool to visualise cells in mice. Light of one wavelength illuminates the specimen; a shift in wavelength (emission) is detected by charge couple device (CCD) camera. To minimise autofluorescence imaging in the near infrared is recommended, this also maximises tissue penetration. Fluorochromes can be conjugated to antibodies for specific targets as well using tumour cells transfected with fluorescent protein (Folli et al., 1994, Yang et al., 1999).

BLI is used to detect photons emitted from cells genetically engineered to express luciferase. BLI is a sensitive and quantitative method allowing molecular and cellular events involved in tumourigenesis to be monitored. BLI can rapidly reveal therapeutic efficacy in pre-clinical studies of novel therapeutics. The most commonly used bioluminescent reporter gene has been luciferase from the North American firefly (Photinus pyralis, luc). Luciferase catalyses the conversion of the substrate d-luciferin to oxyluciferin in an ATP-dependent manner, leading to emission of photons which are detected by a CCD camera or luminometer in vitro. BLI of cancerous tissue requires the luciferase-encoding gene be transferred to cells or tissues. Cells stably expressing luciferase are then injected into
animals and imaged externally. Some of the earliest experiments testing BLI were mouse xenograft models of human cancers implanted into immunodeficient mice. One such study involved monitoring tumour growth in SCID mice, which had been injected subcutaneously, intravenously or intraperitoneally with HeLa cells transfected with luciferase reporter gene. As few as 1 x 10^3 cells were detected following i.p. injection and the bioluminescent signal correlated with the number of cells inoculated (Edinger et al., 1999). BLI is an extremely useful imaging tool as mammalian tissues do not have significant intrinsic bioluminescence. Therefore, bioluminescence is restricted to cells expressing the luciferase reporter gene. Moreover, d-luciferin is not toxic to mammalian cells (Sweeney et al., 1999). BLI is one of the most sensitive technologies available for detection of cancer in small animals. In a gene delivery experiment, a recombinant adeno-associated virus transduction system was used to deliver the luciferase gene under the control of the elongation factor 1 alpha promoter to mice in utero. Long-term expression of luciferase was seen in the liver and peritoneum. Limiting dilution PCR performed 8 months after birth showed that signal intensity generated from 1 Luc+ liver cell in 10^6 Luc- cells was enough to be detected externally (Lipshutz et al., 2001).

Advances in BLI have led to the development of transgenic mouse models expressing luciferase. One such transgenic mouse was generated to strongly express luciferase (PSA-luc) in the epithelium of all 4 lobes of the murine prostate gland under control of the human PSA promoter. Following establishment of mice expressing both PSA-luc and the SV40-antigen T large antigen, spontaneous tumour growth was monitored. As light emission should be proportional to the cellularity of the luciferase-expressing tissue, a relative increase in bioluminescence was indicative of spontaneous tumour development. Ex vivo analysis confirmed the presence of large bioluminescent prostate tumours (Lyons et al., 2006). BLI is fast and relatively inexpensive compared with MRI and PET. Even though BLI is a useful technique in small animal studies it does not transfer to the clinic as BLI can only image tumours expressing a fluorescent or luminescent reporter gene.

Understanding of human cancer biology has been greatly enhanced by the study of mouse models. Current technologies available for monitoring tumour growth in vivo have also contributed greatly to understanding tumour biology and in testing new therapies. All molecular imaging technologies have advantages and disadvantages. The use of multifunctional reporter genes that link two or more imaging modalities will be of great benefit in monitoring cancer. Fusion genes for bioluminescent and fluorescent imaging are currently available (Day et al., 1998). The ability to non-invasively image small animals will greatly enhance early detection of tumours and facilitate evaluation of new therapies.
1.8 Overall aim:
In prostate cancer, the major clinical challenge is the development of androgen independent disease and metastasis. The primary tumour can normally be effectively treated by surgery, chemotherapy, radiotherapy or a combination. However, therapies previously used against the primary tumour may not effect the growth of androgen independent disease and metastases. Therefore, new therapies are required to treat advanced prostate cancer. Therapies targeting the interaction of the host tissue and tumour such as growth factors that promote cell proliferation, migration and angiogenesis may be of value in the treatment of prostate cancer.

The IGF pathway plays a role in cell growth, proliferation and angiogenesis, all of which are important in tumour growth. Due to the involvement of the IGF pathway in prostate cancer, targeting this pathway may prove beneficial in treating prostate cancer.

1.8.1 Specific Aims:
1. To assess membrane and cytoplasmic IGF IR expression in prostate cancer tissue microarrays and its association with Gleason grade.
2. To measure plasma IGF I and PAPP-A levels in prostate cancer patients relative to BPH patients.
3. To investigate the expression and activation of the IGF signalling pathway (IGF IR, IGFBPs, PAPP-A, Akt and ERK) in a human androgen independent prostate cancer cell line, PC-3M-luc2.
4. To purify protease resistant IGFBP 4 (dBP4) from dBP4 transfected HEK 293 cells and to evaluate the effect of dBP4 and TKIs on cell proliferation in vitro using PC-3M-luc2 cells.
5. To assess the effect of dBP4 on tubule formation (angiogenesis) by human endothelial cells in vitro.
6. To examine the effect of inducible vector-expressed dBP4 on prostate cancer growth and metastasis by BLI in subcutaneous, orthotopic (prostate) and experimental metastasis models. In addition, the effect of dBP4 on blood glucose, IGF I levels and activation of the IGF pathway (IGF IR, Akt, ERK and VEGF) was assessed in these models.
Chapter 2

Materials and Methods
2.1 Reagents
All reagents were purchased from Sigma-Aldrich chemical company (MO, USA) unless otherwise stated. All reagents were stored as recommended by the supplier. Reagents in powder-form were prepared in distilled water, unless otherwise stated. Solid reagents were weighed using an electronic balance (XS205 Dual Range, Mettler Toledo, OH, USA) and disposable weigh boats. The pH of all solutions was measured using an AP5 pH meter (Denver Instruments Company, CO, USA) calibrated with standards at pH 4.0, pH 7.0 and pH 10.0. Volumes of 0.5 μl - 1000 μl were delivered using calibrated pipettes (Gilson S.A, France). Volumes from 1 - 20 ml were measured using disposable plastic pipettes. Volumes in excess of 20 ml were measured using graduated cylinder.

2.2 Cell Culture
All tissue culture was carried out using aseptic technique in a Heraeus Class II A laminar airflow hood. The hood was wiped down with 70 % (v/v) ethanol (EtOH) before and after use and cleaned on a regular basis with 70 % (v/v) EtOH and MicroSol (Anachem Ltd, UK).

2.2.1 Cell lines
The human androgen independent prostate cancer cell line PC-3M-luc2 (PC-3M cells transfected with luciferase) was purchased from Caliper Life Sciences (MA, USA) and was maintained in Modified Eagle Medium (MEM, Sigma-Aldrich, MO, USA) supplemented with 10 % (v/v) FCS (fetal calf serum, Biosera, East Sussex, UK). The human embryonic kidney cells (HEK 293) and HEK 293T (contains SV40 Large T antigen) cells were obtained from the American Type Culture Collection (ATCC Md, USA) and maintained in high glucose (4.5 g/L) Dulbecco’s Modified Eagle Medium (DMEM, Biosera, East Sussex, UK) supplemented with 10 % (v/v) FCS. Cell lines were grown in 75 cm² flasks (Sarstedt, Nubrecht Germany) with filter caps and were incubated at 37°C in a humidified atmosphere of 5 % CO2 and 95 % air (NuAire™IR Autoflow CO2 incubator, MN, USA).

2.2.2 Subculture of PC-3M-luc 2 cells, HEK 293 and HEK 293T cells
All cell lines were checked daily using an inverted microscope (Nikon Eclipse TS100, Micron Optical, Wexford, Ireland). Once cells reached 70-80 % confluency, they were subcultured. Flasks were removed from the incubator into the laminar airflow hood wiped down with 70 % (v/v) ethanol (EtOH). Cell culture medium, phosphate buffered saline (PBS (0.137 M) with calcium and magnesium, Biosera, East Sussex, UK) and 1x trypsin-ethyldiamine tetraacetic acid (EDTA) (0.05 % (w/v) trypsin, 0.02 % (w/v) EDTA, Biosera, East Sussex, UK) were heated to 37°C in a water bath.
Cell culture medium was removed and the monolayer was washed with 10 ml of pre-warmed PBS. Trypsin-EDTA (2 ml) was then added to the monolayer of cells. The cap was replaced onto the flask and cells were incubated at 37°C until cells detached from the base of the flask. The flask was placed into the laminar airflow hood and 10 ml of pre-warmed medium (containing 10 % (v/v) FCS) was added to each flask. Cells (12 ml) were seeded in fresh flasks at a ratio of 1:6 (2 ml cells per flask) and fresh medium added to a final volume of 13 ml. Cells were maintained as described in 2.2.1.

2.2.3 Preparation of frozen cell stocks
Frozen stocks were prepared from cells at 70 - 80 % confluency in 75 cm² flasks. Cells were harvested as described above (2.2.2). Cells were pelleted (ALC PK 121 Centrifuge, DJB Labcare, UK) at 1100 × g for 3 min. In the laminar airflow hood the supernatant was removed and cells were resuspended in 1.5 ml PBS, layered on top of 1 ml FCS and centrifuged at 1100 × g for 10 min. Supernatant containing cell debris was removed and cell pellet was in itself, made up to 1 ml with FCS and transferred to a 2 ml cryovial (Greiner Bio-One, Frickenhausen, Germany). An equal volume of cryoprotectant (90 % (v/v) FCS, 10 % v/v DMSO (dimethyl sulfoxide)) was added dropwise to the cell suspension with gentle mixing between drops. The cryovial was sealed and then placed at -80°C in a cryo-freezing container (Nalgene Labware, NY, USA) for 24 h and then transferred to a liquid nitrogen tank (Cryomed CRM-2800, Forma Scientific, OH, USA) for long-term storage.

2.2.4 Revival of frozen cells
A vial of frozen cells was removed from liquid nitrogen and thawed rapidly at 37°C. The thawed vial was taken in the laminar airflow hood and wiped down with 70 % EtOH. Pre-warmed culture medium (12 ml) was added to two 75 cm² flasks and 1 ml of cells was added to each flask. The cells were allowed to recover and adhere overnight at 37°C. The following day medium was replaced with fresh pre-warmed medium and flasks were maintained as per section 2.2.1 and 2.2.2.

2.2.5 Cell counting assay
The Trypan-Blue dye (Gibco-BRL, Paisley, UK) exclusion test is based on the ability of viable cells to actively exclude the dye due to an intact membrane whereas dead cells are unable to exclude the dye and appear blue under a microscope. Following trypsinisation of cells, 20 µl of cell suspension was added to an equal volume of Trypan Blue dye and mixed by pipetting. After 2 min, 10 µl was added to a haemocytometer (Brightline model, Sigma-Aldrich, MO, USA) and cells were counted under 20 × magnification.
Cells within the outer 4 x 4 boxes were counted and the mean was used to calculate the cell density.

Cell density was calculated using the following formula:

\[
\frac{N}{4} \times 2 \times 10^4 = \text{cells/ml}
\]

\(N\) = mean cell number per field counted,

4 = number of fields counted,

2 = dilution factor and

10^4 = haemocytometer constant

### 2.2.6 Single cell cloning by serial dilution

A serial dilution method was used to obtain HEK 293 dBp4 clones expressing high levels of protease resistant IGFBP 4 (dBp4). The PAPP-A cleavage site of wildtype IGFBP 4 was previously mutated by site directed mutagenesis from 119-KHMAKVRDIRSKMK-133 to 119-AAMAAVADASAMA-133. To all wells in the 96 well plate except A1 100 µl of medium was added. To well A1 2 x 10^3 HEK 293 dBp4 cells (200 µl) were added. Using a single channel pipettor 100 µl from the first well was transferred into B1 and mixed gently. The 1:1 serial dilutions were continued down the column, discarding 100 µl from H1. An additional 100 µl of medium was added to each well in column 1. Using the pipettor, 100 µl from the wells in the first column was transferred to those in the second column (A2 - H2) with gentle mixing. These 1:1 serial dilutions were carried out across each column making sure to discard 100 µl from the last column (A12 - H12), the final volume was of all wells was 200 µl. Figure 2.1 shows a schematic of the dilutions. Cells were incubated at 37°C in a humidified CO2 incubator. After 4 - 5 days, clones were identified and on day 7 - 10 clones were scored by marking wells that contained a single colony. Single colonies were subcultured into 24 well plate. dBp4 expression by clones was assessed by Western blot (Section 4.2.2)

### 2.2.7 Mycoplasma Testing

#### 2.2.7.1 MycoAlert Assay

Mycoplasma testing was carried out monthly using a commercial MycoAlert® (Lonza, Basel, SWI) mycoplasma detection assay. This test exploits the reaction of mycoplasma enzymes with MycoAlert® Substrate catalysing the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). Increased levels of ATP due to the presence of Mycoplasma can be detected using luciferase bioluminescence, emission read at 565 nm.
Figure 2.1: Schematic for serial dilutions. $2 \times 10^3$ HEK 293 dBp4 cells were added to A1 and diluted 1:1 down column 1, then 1:1 across each column as shown.
ATP + Luciferin + O₂  \[\rightarrow\]  Oxyluciferin + AMP + PP₁ + CO₂ + Light + Mg²⁺

The ratio of ATP before and after addition of MycoAlert is indicative of presence or absence of *Mycoplasma*. Cell culture supernatant (2 ml) was added to a 15 ml tube and cell debris was pelleted by centrifugation at 1100 × g for 5 min. Cleared supernatant (100 μl) was transferred to a white walled 96 well plate. All solutions were allowed to equilibrate to room temperature. The Luminometer (Victor X Multilabel Plate reader, Perkin-Elmer, Oy, Finland) was programmed to take a 1s integrated reading. MycoAlert® Reagent (100 μl) was added to each sample and incubated at room temperature for 5 min. A₆₅₅ was measured in the Luminometer (Reading A). MycoAlert® Substrate (100 μl) was then added to each sample and incubated at room temperature for 10 min. A₆₅₅ was measured in the Luminometer (Reading B). The ratio of Reading B: Reading A was calculated. A ratio of < 1 indicated absence of *Mycoplasma*. If the ratio was > 1 this indicated samples were *Mycoplasma* positive. For samples giving a borderline ratio (0.9-1.1) a mycoplasma PCR (polymerase chain reaction) was carried out. *Mycoplasma* positive cells were discarded.

2.2.7.2 Mycoplasma PCR

Samples with a borderline ratio in the MycoAlert® test and cells grown in antibiotics were tested using the PCR Mycoplasma Test Kit II (Promocell, Heidelberg, Germany). PCR method allowed identification of low levels of *Mycoplasma* infection. Cell culture supernatant (1 ml) was added to a 15 ml tube and cell debris pelleted at 1100 × g for 5 min. The cleared supernatant was transferred into a 1.5 ml Eppendorf tube (Eppendorf International, Hamburg, Germany) and *Mycoplasma* pelleted (Eppendorf 5417R, Eppendorf International, Hamburg, Germany) at 14, 000 × g for 10 min. Supernatant was discarded and the pellet was resuspended in 50 μl of Buffer Solution and heated at 95°C for 3 min. In a separate tube 35 μl distilled water, 10 μl Reaction Buffer (containing primers, dNTPs, Taq Polymerase) and 5 μl of denatured sample or 1 μl positive control were added. A negative control containing only buffer was also set up. All samples were placed in a PCR thermocycler (PTC-100, Pegasus Scientific Inc., MD, USA) and subjected to 1 cycle at 94° for 30 s followed by 35 cycles of 94°C for 30 s, 60°C for 120 s and 72°C for 60 s followed by 1 cycle of 94°C for 30 s, 60°C for 120 s and 72°C for 5 min. Samples (20 μl) were analysed on a 2 % (w/v) agarose gel with Ethidium bromide (EtBr 0.5 μg/ml). Amplification of *Mycoplasma* resulted in a 270 bp PCR product.

2.3 Tissue Microarray (TMA) Construction

Archival, formalin fixed paraffin embedded (FFPE) prostate tumour samples from patients on file within Beaumont Hospital, Dublin were selected by Tony O'Grady. Ethics approval was
awarded as part of the Prostate Cancer Research Consortium. All FFPE blocks were sectioned, haematoxylin and eosin (H & E) stained and graded by a pathologist (Prof. Elaine Kay) to confirm stage and grade of the tumours. The relevant tumour areas were marked and used for TMA construction. TMAs were constructed from tissue blocks using the Beecher Instruments R Tissue Microarrayer (Beecher Instruments Inc, WI, USA). Three 1 mm cores of varying Gleason grades and three 1 mm cores of benign prostatic hyperplasia (BPH) were selected from each case of prostate carcinoma.

2.3.1 Immunohistochemical staining of TMAs

2.3.1.1 IGF IRβ staining of TMAs

4 µm sections from all TMAs were prepared for immunohistochemistry. Sections were immunostained with IGF IRβ rabbit polyclonal antibody (Cell Signalling Technology, MA, USA) at 1:100 dilution on Bond Max Automated Immunohistochemistry system (Leica Microsystems GmbH, Wetzlar, Germany). TMAs were deparaffinised and pre-treated with the Epitope Retrieval Solution 2 (EDTA-buffer pH8.8) at 98°C for 20 min. After washing steps (Bond wash buffer (Leica Microsystems GmbH, Germany)), peroxidase blocking was carried out for 10 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH, Germany). Tissues were again washed and then incubated with the IGF IRβ (diluted in Bond antibody diluent, Leica Microsystems GmbH, Germany) for 30 min. Subsequently, tissues were incubated with anti-rabbit secondary conjugated with dextran polymer for 10 min and developed with DAB (Diaminobenzidine)-Chromogen for 10 min. All sections were counterstained with haematoxylin. For negative controls, primary antibody solution was omitted. Breast cancer tissue was used as a positive control. Each tissue microarray was scored based on staining strength in either the membrane or cytoplasm (0 negative, +1 weak, +2 moderate, +3 strong).

2.3.1.2 Haematoxylin and Eosin staining of TMAs

Briefly, cut sections were subjected to on-board dewaxing (Dewax solution - Vision BioSystems™). Slides were stained in Haematoxylin for 2 min, rinsed in water followed by addition to acid alcohol for 2 s (0.1 % (v/v) HCL in 50 % (v/v) EtOH). Slides were rinsed in water for 15 min, stained with Eosin for 1 min, rinsed in 3 changes of water. Slides were dehydrated in 95 % (v/v) EtOH for 1 min followed by 100 % (v/v) EtOH for 1 min, rinsed in xylene twice for 5 min then coverslipped.

2.4 CD31* staining of human endothelial cells

Dr. Constanze Schadow carried out analysis of tubule formation in response to dBP4 treatment. An Angiokit was purchased from TCS Cell works (Buckingham, UK). The kit...
contained growing human endothelial cell cultures at the earliest stages of tubule formation in a 24 well plate format. This allowed for analysis of tubule formation (angiogenesis). Tubules were stained for CD31+ an endothelial cell adhesion molecule. Medium was aspirated from each well and washed with 500 μl of wash buffer (PBS). Cold fixative (500 μl, 70 % (v/v) EtOH) was added to each well and incubated at room temperature for 30 min. The fixative was decanted and wells were washed 3 times with 500 μl of blocking buffer (PBS/1 % (w/v) BSA). The final wash was carefully decanted. The primary antibody (mouse anti-CD31+) was diluted 1:400 in blocking buffer. 500 μl/well of primary antibody was incubated for 1 h at 37°C. The plate was washed 3 times with 500 μl/well blocking buffer. Secondary antibody (goat anti-mouse IgG alkaline phosphatase conjugate) was diluted 1:500 in blocking buffer. 500 μl/well of the secondary antibody was added and incubated for 1 h at 37°C. The plate was washed 3 times with 500 μl/well dH2O. Two 5-bromo-4-chloro-3'-indolylphosphate/nitro-blue tetrazolium (BCIP/NBT) tablets were dissolved in 20 ml dH2O and filtered through a 0.2 μm filter. 500 μl/well of substrate was added and incubated at room temperature until tubules developed a dark purple colour (3-10 min). The plate was washed 3 times with 500 μl/well dH2O. The final wash was discarded and the wells were air dried. Tubule formation was assessed with TCS Cell Works Angiosys Imaging Analysis software (Buckingham, UK).

2.5 RNA and DNA analysis

2.5.1 Isolation of RNA

As RNA is susceptible to degradation by RNases which are ubiquitous in the laboratory, all materials for RNA isolation were autoclaved at 121°C for 20 min. The bench and pipettes to be used were wiped down with RNAse Zap (Applied Biosystems, TX, USA). Trizol® (1ml, Invitrogen, CA, USA) was added to a 75 cm² flask, cells were incubated on a shaker table for 5 min. After cells were fully lysed, the Trizol® was transferred to a 2 ml Eppendorf tube. For every ml of Trizol® 270 μl chloroform was added, the tube was mixed vigorously for 15 s, then incubated at room temperature for 5 min. Phases were separated by centrifugation at 12,000 × g for 15 min. The aqueous (top) layer was placed into a fresh tube. RNA was precipitated by adding 670 μl isopropanol, mixed and incubated at room temperature for 10 min. RNA was pelleted by centrifugation at 12,000 × g for 10 min at room temperature. Supernatants were carefully decanted and pellets washed with 1 ml 75 % (v/v) EtOH (in DEPC (diethylpyrocarbonate) treated water. RNA was pelleted by centrifugation at 12,000 × g for 10 min at 4°C. Supernatant was decanted, the pellet was air dried for 5 min and then resuspended in 50 μl TE Buffer (10mM Tris-HCl (pH 7.4), 1mM EDTA Buffer (pH 8.0)). Samples were kept on ice from this stage.
2.5.2 RNA/DNA quantification

A nanodrop (ND-1000 UV-Vis Spectrophotometer, NanoDrop Technologies, DE, USA) was used to quantify RNA/DNA. 1 µl of distilled water was used as a blank, followed by 1 µl TE buffer. Once the nanodrop was at zero, 1 µl of RNA/DNA sample was added with software (ND-1000) set to RNA 40 / DNA 50. A ratio of 1.8 - 2.0 indicates pure RNA/DNA, below 1.8 may indicate the presence of protein and above 2 may indicate the presence of organic solvents. Using the Beer-Lambert law it is possible to relate the amount of light absorbed to the concentration of the absorbing molecule. At a wavelength of 260 nm, the extinction coefficient for double-stranded DNA is 50 (µg/ml)⁻¹ cm⁻¹; for single-stranded DNA and RNA it is 38 (µg/ml)⁻¹ cm⁻¹.

Concentration of double stranded DNA = absorbance at 260 nm × 50 × dilution

Concentration of single-stranded DNA and RNA = absorbance at 260 nm × 38 × dilution

2.5.3 cDNA synthesis

cDNA was produced from total RNA. 1 µg total RNA was digested with 1U DNase I (Invitrogen, CA, USA) in a total volume of 10 µl at room temperature for 15 min (to degrade any contaminating DNA present in the RNA sample). DNase I was inactivated by the addition of 1 µl 25 mM EDTA (Invitrogen, CA, USA) and heating to 70°C for 10 min. cDNA was synthesised from total RNA using the Superscript II First-Strand Synthesis System for reverse transcriptase PCR (RT-PCR, Invitrogen, CA, USA). To the DNase treated RNA, 4 µl 5 x First Strand Buffer, 2 µl 0.1 M DTT, 1 µl random primers and 200 U Superscript II reverse transcriptase (RT) (M-MLV) was added. cDNA synthesis was carried out at 42°C for 50 min and RT inactivated at 70°C for 15 min, then stored at -20°C.

2.5.4 Primer Design

Primers were designed based on published mRNA sequence data and Genbank data. Where possible each primer was designed with 50 % G - C content to maximise primer annealing to template without an excessively high annealing temperature. This allows stable binding of primer to cDNA as G - C base pairs contain three hydrogen bonds, whereas A - T only contains two. Sequence complementarity between primer pairs was avoided so as to prevent the formation of primer dimers. Primer Blast was used to design primers (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Selected primer sequences were compared to published sequences (Genbank) using BLAST (http://www.ncbi.nih.gov/BLAST) to ensure there was no cross reactivity with sequences other than the desired mRNA. Primers were synthesised by Eurofins MWG Operon (Ebersburg, Germany) and LGC Genomics (Berlin, Germany).
PAPP-A specific primers:
Forward primer: 5' AGGTGGCATTGTCTTGAACC 3'
Reverse primer: 5' AAGCCACAGGTGTCATTTCC 3'

β-actin specific primers
Forward primer: 5' TGTGATGGGTGGGAATGGGTGAG 3'
Reverse primer: 5' TTTGATGTCACGCACGATTTCC 3'

β-actin primers were purchased from Stratagene (CA, USA).
Primer sequences for cloning protease resistant IGFBP 4 (dBP4) into tetracycline inducible lentiviral vector (pLVX Tight Puro)
Forward primer: 5' GGGGATCCAAAGGAGATATACCATGCTGCCCT 3'
The underlined sequence is the BamHI restriction enzyme site, the sequence in bold is the start codon. The italicised sequence is dBP4 sequence. The sequence AAAGGAG is the ribosomal binding site and TACCATGG is the Kozak sequence.
Reverse primer: 5' GGGAATTCTTAGTGATGGTGATGGTGATGGTG 3'
The underlined sequence is the EcoRI restriction enzyme site, the sequence in bold is the stop codon and the normal text is the sequence for the His-tag (6 × histidine codons) which allows identification of the recombinant dBP4 protein by Western blotting.

2.5.5 Polymerase chain reaction (PCR)
cDNA or plasmid DNA was used as a template for PCR. A master mix was aliquoted to each reaction tube before template was added. Each reaction contained 5 μl 10 × PCR Buffer; 2 μl 25 mM MgCl2, 1 μl 10 mM dNTPs, 0.5 μl Taq DNA Polymerase (5U/μl, Fermentas, Germany), 1.5 μl cDNA and 38 μl dH2O. No template was added to negative controls, cDNA from HEK 293 cells was used as a positive control for PAPP-A PCR and pTriEx4 Neo dBP4 (Appendix 1) plasmid was used as positive control for colony PCR.

Amplification of PAPP-A:
cDNA generated from RNA isolated from PC-3M-luc2 and HEK293 cells was amplified using PAPP-A primers. cDNA was denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 58°C for 45 s (primer annealing), 72°C for 45 s (primer extension) followed by 72°C for 10 min.

Amplification of β-actin:
cDNA generated from RNA isolated from PC-3M-luc2 and HEK293 cells was amplified using β-actin primers. cDNA was denatured at 94°C for 5 min, primers annealed at 60°C for 5 min, primer extension at 72°C for 90s followed by 35 cycles of 94°C for 45s, then 60°C for 45s and 72°C for 90s.
Amplification of dBP4 for cloning into tetracycline inducible lentiviral vector (pLVX Tight Puro)

dBP4 was amplified from 1 ng of pTriEx4 Neo dBP4 for subcloning into pLVX Tight Puro lentiviral vector. Using the forward and reverse primers PCR was carried out, 94°C for 90 s, 50°C for 30 s, 72°C for 2 min, 30 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 2 min and 72°C for 10 min.

Amplification of IGFBPs and IGFs by Multiplex PCR

cDNA generated from RNA isolated from PC-3M-luc2 cells was amplified using a Multiplex PCR human IGF and IGFBPs Kit (Maxim Biotech, CA, USA) as per the manufacturer’s instructions which were 2 cycles of 96°C for 60 s followed by 63°C for 4 min and 35 cycles at 94°C for 60 s, 63°C for 2 min and 70°C for 10 min.

PCR products from PAPP-A, β-actin and Multiplex PCR were fractionated on a 2 % (w/v) 1 x TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) agarose gel containing 0.5 μg/ml EtBr (Sigma-Aldrich, MO, USA). Products from dBP4 PCR were fractionated on a 0.8 % (w/v) TAE low melting point agarose gel containing 0.5 μg/ml ethidium bromide.

2.5.6 DNA Electrophoresis

DNA was fractionated on a non-denaturing 0.8-2 % (w/v) agarose gel in 1 x TAE. Where DNA was to be recovered, low melting point (LMP) agarose (Invitrogen, CA, USA) was used. Agarose (0.4 - 1 g) was added to 50 ml 1 x TAE and dissolved in the microwave then to this 5 μl of ethidium bromide (10 mg/ml) was added to the molten agarose. The agarose was mixed and poured into a gel tray with well forming comb in place and allowed to set at 4°C. The comb was then removed, 1 kb DNA molecular weight marker (5 μl, Fermentas, Germany) was loaded alongside samples. All samples were mixed with appropriate amount of 6 x loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF in 30 % (v/v) glycerol (Sigma-Aldrich, MO, USA), distilled water) before loading into wells. Electrophoresis was carried out at 100 V for 1 h or until the dye front was 2/3 way down gel depending on size of fragments. DNA was visualised using a UV transilluminator (TFX-20M UV illuminator, Vilber Lourmat, Marne-la-Vallée, France). Gels were photographed using Genesnap (Syngene, MD, USA).

2.6 Molecular cloning

Molecular cloning, A Laboratory Manual (Sambrook & Russell, 2001) was used as a guide for cloning methods. Sections 2.5.4-2.5.6 have already described the methods used to amplify dBP4 insert. Following DNA electrophoresis, the dBP4 PCR product was recovered from the agarose gel.
2.6.1 DNA recovery from agarose

The desired DNA bands were visualised using a UV transilluminator (TFX-20M UV illuminator) and excised from LMP agarose with a clean sterile scalpel. The agarose slice was transferred into a pre-weighed 1.5 ml tube. The tube was weighed again after addition of the gel slice. GeneJet Gel Extraction Kit (Fermentas, Germany) was used to extract the DNA from the gel. A 1:1 (weight: volume) volume of binding buffer was added to the gel slice (100 mg: 100 µl binding buffer). The slice was liquefied at 60°C for 10 min and added to a GeneJet purification column. The column was centrifuged at 14,000 x g for 1 min and flow through was discarded. Wash buffer (700 µl) was added to the column, then centrifuged at 14, 000 x g for 1 min, flow through discarded and the empty column was centrifuged for a further 1 min. The GeneJet column was placed into a clean 1.5 ml Eppendorf tube, 50 µl elution buffer was added to the centre of the column and DNA was eluted at 14, 000 x g for 1 min. DNA quantification was carried out as described in section 2.5.2.

2.6.2 DNA digestion with restriction enzymes

All fast digest restriction enzymes were purchased from Fermentas (Fermentas, Germany). Double digests were carried out together in Fast Digest reaction buffer (Fermentas, Germany). Each reaction contained 5 µl 10 x Fast Digest Reaction Buffer; 0.5 µg DNA, 10 U EcoRI, 15 U BamHI, 1 µl alkaline phosphatase and sterile distilled water to a final volume of 50 µl. Restriction enzymes were added after all other reaction components were mixed well and then DNA was digested at 37°C for 40 min. Digested DNA was fractionated by electrophoresis through LMP agarose gel (2.5.6) and purified as described (2.6.1). Purified DNA was precipitated (2.6.3) and quantified (2.5.2).

2.6.3 Ethanol precipitation of DNA.

DNA was precipitated at -20°C for 30 min by addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100 % (v/v) EtOH based on the combined volume of DNA and sodium acetate: DNA was pelleted at 14,000 x g for 20 min. Supernatant was decanted and the DNA pellet was washed with 70 % (v/v) EtOH, air dried for 5 min and resuspended in TE buffer.

2.6.4 DNA ligation

DNA was ligated at a molar ratio of 10:1 and 20:1 insert (5 ng/ml) to vector DNA (5 ng/ml). Vector and insert were mixed with 10 x ligation buffer (Fermentas, Germany) and 5 U T4 DNA ligase (5U/µl, Fermentas, Germany) in a final volume of 20 µl. Controls for self ligation contained vector DNA only or a ligation control containing no T4 DNA ligase. Reactions were incubated for 4 hours at room temperature then placed on ice.
2.6.5 Bacterial transformation

2.6.5.1 Media/Solutions

Ampicillin

100 mg ampicillin (Sigma-Aldrich, MO, USA) was added to 10 ml distilled water, filter sterilised through a 22 μm syringe filter and stored in aliquots at -20°C. Once thawed aliquots were discarded.

LB (Luria Bertani) Broth

LB Broth mix (25 g, Merck, Nottingham, UK) was dissolved in 1 L distilled water and autoclaved. LB broth contains 10 g/L Bacto-tryptone, 5 g/L yeast extract and 10 g/L NaCl, pH 7.5.

LB agar

LB agar mix (35 g, Merck, Nottingham, UK) was dissolved in 1 L distilled water and autoclaved. LB agar contains the same components as the LB broth, but with 15 g/L of agar.

Antibiotic containing LB broth

Before use, ampicillin stock solution (100 mg/ml) was added to LB broth to a final concentration of 100 μg/ml (LBAmp100).

Antibiotic containing LB agar

LB agar prepared earlier was dissolved in the microwave, allowed to cool to 50°C and ampicillin (100 μg/ml) added. LBAmp100 agar plates were prepared by pouring 30 ml agar into sterile petri dishes under aseptic conditions, allowed to set and stored at 4°C.

SOC medium

SOC medium was purchased from Invitrogen (Invitrogen, CA, USA) and stored at room temperature. SOC medium contains 2 g/L tryptone, 0.5 g/L yeast extract, 0.05 g/L NaCl, 250 mM KCl, 1 M glucose and 2 M MgCl₂.

2.6.5.2 Transformation of bacterial cells

One Shot® TOP10 competent E.coli (Invitrogen, CA, USA) were used as the host cells for plasmids and stored at -80°C until use. Cells were thawed on wet ice and 50 μl aliquoted into pre-chilled 1.5 ml tubes. DNA ligation (2 μl) were added to E.coli, incubated on ice for 30 min, then heat pulsed at 42°C for 30 s before cooling on ice for 2 min. SOC medium (250 μl) was added to each tube and incubated at 37°C for 1 h with shaking at 225 rpm. Cells (100 pl) were then spread on LBAmp100 agar and incubated inverted overnight at 37°C.
2.6.6 Colony PCR
Bacterial colonies were screened for the presence of insert (dBP4) by colony PCR. Individual colonies (28) were spotted onto a gridded LBAm100 agar plate with a sterile pipette tip and the remainder of the colony added to 5 μl (5% v/v) Triton X-100 (Merck, Nottingham, UK) and lysed at 95°C for 5 min, then used as template for PCR (2.4.5).

2.6.7 Plasmid purification from bacterial cultures
Single bacterial colonies selected from LBAm100 agar were inoculated into LBAm100 broth (10 ml in 50 ml polypropylene tube) and grown overnight at 37°C with shaking. For small scale purification (10 ml) Qiaprep Spin Miniprep Kit (Qiagen, MD, USA) was used. For large scale purification (100 ml culture) Qiagen Endofree Maxi Prep system (Qiagen, MD, USA) was used. The maxi prep kit was also used when preparing DNA for transfection to remove bacterial endotoxins, which interfere with transfection efficiency. Purification protocols were as per manufacturer’s instructions.

2.6.8 Sequencing of cloned inserts
All sequencing was carried out by LGC Genomics (Berlin, Germany) single read sequencing. Primers were designed to span the vector insert junctions (Section 2.4.4). Sequence data was compared to published sequences (Genbank) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). If deviations from expected sequences were identified samples were re-sequenced.

2.7 Mammalian cell transfection and transduction
2.7.1 Transfection of HEK 293 T cells with pLVX pTet-On Advanced and pLVX Tight Puro dBP4
All work was carried out in a class II facility. HEK 293 T cells were used as packaging cells to produce virus particles for transduction of PC-3M-Luc2. Figure 2.2 shows a flow diagram of the steps involved in generating cells expressing dBP4 under the control of a Tet inducible promoter. HEK 293 T cells were transfected with pLVX pTet-On Advanced (3 μg, Appendix 2) or pLVX Tight Puro dBP4 plasmids (3 μg, Appendix 3), psPAX2 plasmid (3 μg, viral packaging, Appendix 4) and pmD2.g plasmid (2.5 μg, envelope, Appendix 5) using Genejuice (Merck, Nottingham, UK) in 600 μl OptiMEM (Gibco-BRL, Paisley, UK). Transfection reaction was mixed and incubated at room temperature for 10 min, then added to 70% confluent HEK 293 T cells in 25 cm² flasks and incubated at 37°C overnight. The following day the medium was replaced with fresh pre-warmed medium and incubated overnight to increase viral titers.
2.7.2 Transduction of PC-3M-luc2 with pLVX pTet-On Advanced and pLVX Tight Puro dBP4

Medium containing virus particles from the HEK 293 T cells was filtered through a 0.45 μm syringe filter to remove any cellular debris then added to 70 % confluent PC-3M-luc2 cells in a 25 cm³ flask and incubated at 37°C overnight. The following day, medium was replaced with pre-warmed fresh medium, cells were allowed to recover for 3 days. PC-3M-luc2 cells were first transduced with pLVX pTet-On Advanced plasmid and clones selected by incubating in 400 μg/ml G418 (geneticin, Gibco-BRL, Paisley, UK) for 4 weeks. Once stable clones were established the pTet-On PC-3M-luc2 cells were then transduced with the pLVX Tight Puro dBP4 plasmid and clones were selected by incubating cells in 2 μg/ml puromycin (Sigma-Aldrich, MO, USA) for 7 days. dBP4 expression by selected clones was induced by 0.1 μM Doxycycline to culture medium for 3 days and followed by Western blot analysis of conditioned medium.

2.8 Luciferase assay

A 200 x luciferin stock solution (30 mg/ml) was prepared in sterile water. A 1 x stock (150 μg/ml) was prepared in fresh cell culture medium. The 1 x stock was added to cells for 2 min at 37 °C. Cells were then imaged using the IVIS Spectrum.

2.9 Protein analysis

2.9.1 Collection and storage of cell lysates and conditioned medium

Cell lysates were prepared from 6 well plates. Medium was discarded and cells were washed with PBS (3 ml/well). RIPA Buffer (200 μl/well, 25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% v/v NP-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS buffer, Sigma, MO, USA) containing 2 μl of 100 x protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin-A, E-64, bestatin, leupeptin and aprotinin, Merck, Nottingham, UK) and 2 μl 100 x phosphatase inhibitor cocktail (sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole, Sigma, MO, USA) was added. Cells were scraped into 0.5 ml tubes, lysed by pipetting, and incubated on ice for 30 min and debris was pelleted at 14,000 x g for 10 min. Lysates were stored at -80°C until use and thawed on ice.
Figure 2.2: Flow diagram of generation of PC-3M-luc2 with tetracycline (Tet) inducible vector expressing dBP4. The diagram shows the steps taken to create the Tet inducible PC-3M-luc2 cells. HEK293 T cells were transfected with the pTet-On Advanced vector along with psPAX2 (viral packaging) and pmD2.g (viral envelope) to produce virus particles. PC-3M-luc2 cells were transduced with the virus particles containing the pTet-On Advanced vector and selected in geneticin (400 µg/ml) for 4 weeks to produce stable clones. HEK 293 T cells were used to package the pLVX Tight Puro dBP4 vector as before. pTet-On PC-3M-luc2 cells were transduced with virus particles containing pLVX Tight Puro dBP4 and stable clones selected in puromycin (2 µg/ml) for 7 days. Doxycycline was added to cells to test for induction of dBP4. Doxycycline interacts with reverse transcriptional transactivator (rtTA) and binds to the tetracycline response element (TRE mod) switching on dBP4 by use of the CMV (cytomegalovirus) promoter.
Serum-free culture medium (conditioned medium) was collected from 75 cm² flasks (10 ml) after 48 h. Conditioned medium was concentrated using 3 kDa Centricon YM-3 filters (Millipore, MA, USA) according to manufacturer's instructions. Once a volume of 0.5 ml or less was obtained 5 µl of 100 x protease inhibitor cocktail and 100 x phosphatase inhibitor cocktail was added and conditioned medium was stored at -80°C until use and thawed on ice.

2.9.2 Protein quantification using Bio-Rad DC protein assay

Bio-Rad DC (detergent compatible) protein assay was purchased from Bio-Rad (Bio-Rad, CA, USA). The assay is based on the reaction of protein with an alkaline copper tartrate solution (Reagent A) and Folin reagent. There are two steps that lead to colour development, the reaction between protein and copper in an alkaline medium and the subsequent reduction of Folin reagent by the copper-treated protein. Colour development is primarily due to the amino acids tyrosine and tryptophan. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, producing one or more of several possible reduced species with a characteristic blue colour. The maximum absorbance ($\lambda_{max}$) is 750 nm. Using a standard curve of known protein concentrations (0.125 - 2 mg/ml, bovine serum albumin (BSA)-diluted in RIP A buffer or serum free medium) the protein concentration of unknown samples can be determined through comparisons of optical densities of known protein concentrations. Sample or standard (5 µl) were added to a 96 well plate in duplicate. Working reagent, 20 µl Reagent S (detergent) (25 µl/well) in 1 ml Reagent A (alkaline copper tartrate solution) was added followed by 100 µl/well Folin solution (Reagent B). Plates were incubated for 15 min at room temperature. Optical density of samples and standards at 750 nm was determined using the Victor X Multilabel Plate reader (Perkin-Elmer, Oy, Finland). A linear standard curve was generated from the standards and the equation of the line was used to calculate the protein concentration of the unknown samples (See Appendix 7).

2.9.3 Immunoprecipitations

Sepharose G beads were washed twice with 1 x TBS (25 mM Tris, 150 mM NaCl, Sigma, MO,USA) at 664 x g for 30 s. Antibodies to be used (Appendix 8) were coupled to Sepharose G beads on a rotating platform overnight at 4°C. Cell lysates were prepared as per section 2.7.1 and 2.7.2. Sepharose G beads were pelleted at 664 x g for 30 s and supernatant was discarded. Cell lysate (250 µg) was added to the antibody coupled Sepharose G beads on a rotating platform for 2 h at 4°C. Beads were pelleted at 664 x g for 30 s, supernatant removed and beads washed 3 times with RIP A buffer. Dry beads were then stored at -20°C until use and thawed on ice when needed.
2.9.4 Protein purification

2.9.4.1 Nickel agarose purification of recombinant dBP4

HEK 293 cells were transfected with a construct containing dBP4 tagged with 6 histidine residues (6 x His-tag) at the C-terminal end. As dBP4 is secreted, conditioned medium was collected from the HEK 293 cells and concentrated 20 fold.

Qiagen Superflow Ni-NTA agarose (Qiagen, MD, USA) bead slurry (3 ml) was loaded onto Bio-Rad disposable chromatography columns and compacted to 1.5 ml by washing with distilled water (30 ml) and equilibrated with 1 x Binding Buffer (15 ml, 300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole). Conditioned medium (500 ml) was loaded on column and allowed to flow through. Beads were washed with 1 x binding buffer (45 ml), followed by 1 x wash buffer (10 ml, 300 mM NaCl, 50 mM sodium phosphate, 20 mM imidazole). Finally, His-tagged dBP4 was eluted from the column using 1 x elution buffer (10 ml, 300 mM NaCl, 50 mM sodium phosphate, 250 mM imidazole). Samples were taken at each step to check for loss of dBP4 during the purification. Eluate was concentrated to a final volume of 500 pi using Centricons YM-3, purified by FPLC (fast protein liquid chromatography).

2.9.4.2 Fast protein liquid chromatography (FPLC)

Following purification of dBP4 with nickel agarose beads, FPLC was used to further purify the dBP4. A Superdex 200 column (GE Healthcare, Bucks, UK) was attached to the FPLC, to purify protein by size exclusion. Based on the size of dBP4 (28 kDa) it was estimated using the user manual, that after 12 ml of running buffer (10 mM HEPES, 150 mM NaCl and 1 mM CaCl₂, pH 7.5, Sigma, MO, USA) dBP4 should elute from the column. FPLC was cleaned with 50 ml sterile water followed by 50 ml 20 % (v/v) EtOH. Protein samples were loaded into the FPLC using a syringe and fractions (500 μl) collected in 1.5 ml tubes. All samples were analysed by Western blot to check for dBP4.

2.9.5 PAPP-A and PSA digest of dBP4

Concentrated conditioned medium (20 μl) containing dBP4 was incubated with 2 μl recombinant PAPP-A (conditioned medium from HEK 293 cells transfected with human PAPP-A) (a gift from Claus Oxvig, University of Aarhus, Denmark) and 4 μl IGF II (50 ng/μl) at 37°C overnight. For PSA digestion, 20 μl concentrated conditioned medium was incubated with 2 μl PSA (10 ng/μl) at 37°C overnight. dBP4 cleavage by PAPP-A or PSA was assessed by Western blot (2.7.6).
2.9.6 Western blotting of proteins

Proteins were fractionated by electrophoresis through 4 - 20 % (w/v) pre-cast SDS-PAGE (polyacrylimide gel electrophoresis) gel (Pierce, IL, USA). Gel rigis (Bio-Rad, MA, USA) were washed with distilled water and assembled according to manufacturer's instructions. Gels were placed in the gel rig and chamber and then wells were rinsed with electrode buffer (0.1 M Tris, 0.1 M HEPES, 3 mM SDS, pH 8, Pierce, IL, USA, Appendix 6). Once rinsed 400 ml of electrode buffer was poured into the chamber. Up to 50 μg of protein plus the appropriate amount of 6 x Laemmli buffer (Appendix 6) were denatured at 98°C for 10 min. Samples were loaded on to the gel (35 pl/well) and electrophoresis was carried out at 130 V for 45 min or until the bromophenol dye front reached the end of the gel. Pre-stained protein markers (10-250 kDa) (Fermentas, Germany) were loaded on each gel alongside samples.

2.9.7 Silver staining of SDS-PAGE

Following section 2.7.4 and 2.7.5 a silver stain kit was obtained from GE Healthcare and the protocol was followed as per the manufacturer's instructions. Following SDS-PAGE proteins were fixed for 30 minutes in 40% (v/v) EtOH while shaking. The 40% (v/v) EtOH was discarded and the gel was sensitised for 30 min using a sensitising solution (EtOH, 5% (w/v) sodium thiosulphate, sodium acetate, distilled water). The gel was washed 3 times for 5 min each with water.

Silver nitrate solution (2.5 % w/v) was added to the gel for 20 min with shaking. The silver nitrate solution was discarded and the gel was washed twice for 1 min each with water. Developing solution (0.06 M sodium carbonate, 37% (v/v) formaldehyde) was incubated for 2-5 min depending on how quickly a colour change developed. When the bands of the gel reached the desired intensity the gel was transferred into stop solution (9.8 mM Ethylene Diamine Tetraacetic Acid Disodium (EDTA)) for 10 min with gentle shaking. The stop solution was removed and the gel was washed 3 times for 5 min each. To preserve the gel, 87% (v/v) glycerol was added and incubated for 20 min with gentle shaking.

2.9.8 Western Blot transfer

A wet transfer apparatus (Bio-Rad, CA, USA) was used to transfer proteins to nitrocellulose membrane (Pall Gelman Laboratory, NY, USA). Nitrocellulose and Whatman filter paper were cut to the same size of the gel. The membrane, filter paper and transfer sponges were soaked in 1 x transfer buffer (made from 10 x solution: 1.5M glycine, 0.18M Tris, 0.1% (w/v) SDS; 1 x working solution: 100 ml 10 x solution, 200 ml methanol, made up to a litre with distilled water, Appendix 6) before assembling the transfer apparatus. The transfer sponges were placed within the cassette, which was then overlaid with filter paper, followed by the gel, nitrocellulose (0.2 μm), filter paper and finally another transfer sponge. Air bubbles were
removed and cassettes were clipped together and placed into the wet transfer apparatus with nitrocellulose towards the cathode (-) and gel orientated towards the anode (+) with 1 L of 1 x transfer buffer and a cooling block. Proteins were transferred to nitrocellulose membrane for 1 h at 100V.

2.9.9 Ponceau S staining
To confirm efficient transfer of protein onto nitrocellulose membrane, the membrane was stained with Ponceau S staining solution (Sigma-Aldrich, MO, USA) for 2 min. Protein bands became visible upon destaining with distilled water. Membranes were washed with 1 x TBST (25 mM Tris, 150 mM NaCl, 0.1 % Tween) for 5 min until stain was removed.

2.9.10 Antibody detection for Western blotting
Membranes were incubated with blocking solution (5 % (w/v) fat free dried milk protein, Marvel, Premier Foods, UK) in 1 x TBST (Appendix 6) for 1 h on a shaking table at room temperature. Membranes were washed 3 times for 10 min each with 1 x TBST. The appropriate primary antibody was diluted in either 5 % (w/v) fat free dried milk protein or 5 % (w/v) BSA in 1 x TBST according to the manufacturer's specifications. Primary antibodies (Appendix 8) were incubated overnight at 4°C. Membranes were washed 3 times with 1 x TBST for 10 min each at room temperature. Horse-radish peroxidase (HRP)-conjugated goat anti-rabbit or rabbit anti-mouse antibody (Dako, Glostrup, Denmark) were diluted 1:2000 in 1 x TBST with 5 % (w/v) fat free dried milk protein and incubated for 1 h on a shaking table at room temperature. Membranes were washed 3 times with 1 x TBST for 10 min, followed by 5 min incubation with a chemiluminescent HRP substrate (Pierce ECL Western blotting substrate, IL, USA). The membrane was placed beneath a clear plastic sheet within an X-ray cassette, before being exposed to X-ray film (Kodak Ltd, Herts, UK) for 30 s in the first instance in a dark room under red light. If necessary, the membrane was exposed to fresh film for a shorter or longer time.

2.9.11 IGF binding ELISA
To determine if dBP4 maintains its binding affinity for IGF, an ELISA based method was developed. A 96 well plate was coated with 50 ng/ml IGF I, IGF II and VEGF165 overnight at 4°C. The plate was washed 3 times with 400 μl/well PBST (0.1 % (v/v) Tween) and blocked with 5 % (w/v) BSA in PBS for 1.5 h at 37°C. Blocking solution was removed and the plate was washed 3 times with 400 μl/well PBST. dBP4 (500 ng/ml) was added to each well and incubated for 1.5 h at 37°C. Again the plate was washed 3 times with PBST. Primary antibody, 1:10,000 His-tag HRP (100 μl/well) in 1 % fat free dried milk protein in PBS was
incubated for 1 h at 37°C. The plate was washed 3 times with 400 µl/well PBST. As the primary antibody was HRP-conjugated, 100 µl/well of tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, MO, USA) was added and incubated for 30 min at room temperature. Stop solution, 0.5 M sulphuric acid (100 µl/well) was then added. Absorbance was read at 450 nm on a Victor X Multilabel Plate reader (Perkin-Elmer, Oy, Finland).

2.9.12 VEGF<sub>165</sub> ELISA

To determine VEGF<sub>165</sub> expression by PC-3M-luc2, a VEGF<sub>165</sub> Sandwich ELISA was purchased from R&D Systems (R&D Systems, MN, USA) and was carried out according to the manufacturer's instructions. Briefly, the VEGF<sub>165</sub> ELISA contains an antibody to VEGF<sub>165</sub> immobilised in each well of the 96 well plate. Conditioned medium and VEGF<sub>165</sub> standards (0 - 1000 pg/ml) were added to each well for 2 h at room temperature. The 96 well plate was washed 3 times with 400 µl/well wash buffer and incubated with VEGF<sub>165</sub> conjugate for 2 h at room temperature. The plate was washed 3 times with 400 µl/well wash buffer, before Substrate Solution (100 µl/well) was added and incubated for 20 min in the dark at room temperature. Stop Solution (100 µl/well) was then added (blue to yellow colour change) and absorbance at 570 nm was measured. The OD value for the blank control was subtracted from each sample. A standard curve was constructed which allowed for unknown samples to be determined (Appendix 9). Total protein content was measured as per section 2.7.2 in cell lysates and VEGF<sub>165</sub> levels were normalised to total protein content within the cell (pg/mg).

2.9.13 IGF I ELISA

IGF I in prostate cancer patient plasma samples (obtained from the Prostate Cancer Research Consortium), mouse serum and tumour lysates was measured by ELISA according to the manufacturer's protocol (R&D systems, MN, USA). All samples were assayed in duplicate. Standards for human IGF I (6, 3, 1.5, 0.75, 0.375, 0.188, 0.094 ng/ml) and mouse IGF I (2, 1, 0.5, 0.25, 0.125, 0.062, 0.031 ng/ml) were prepared by serial dilutions of a 60ng/ml or 2 ng/ml stock, respectively. Prostate cancer plasma samples used for the IGF I ELISA were first pre-treated with an acidic dissolution solution, in order to release all IGF I bound to IGFBPs. The 96-well plate supplied was pre-coated with anti-IGF I antibody. Samples (or standard) (50 µl/well) were added in duplicate to each well and incubated for two hours at 4°C. The liquid was aspirated from each well and washed 4 times with 400 µl/well wash buffer supplied with the kit. Cold conjugate (100 pl/well) was added to each well and then incubated for 1 h at 4°C. The liquid was aspirated from each well and washed 4 times with 400 µl/well wash buffer. Substrate solution (100 µl/well) was added to each well and incubated for 30 min at room temperature, while ensuring the plate was protected from light. Stop solution (100 µl/well) was added and a colour change was
observed (blue to yellow). The OD<sub>450</sub> was then determined using a Victor X Multilabel Plate reader (Perkin-Elmer, Oy, Finland). A standard curve was plotted (A<sub>450</sub> vs. concentration) and used to determine the IGF I concentration of the unknown samples (Appendix 10 & 11).

2.9.14 PAPP-A ELISA
PAPP-A levels were measured in prostate cancer patient plasma samples (obtained from the Prostate Cancer Research Consortium) by ELISA according to the manufacturer’s instructions (R&D Systems, MN, USA). All samples were assayed in duplicate. Standards for PAPP-A (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 ng/ml) were prepared by serial dilutions of a 500 ng/ml stock. The supplied 96 well plate was pre-coated with a monoclonal antibody to PAPP-A. Samples and standards (50 μl/well) were added to the plate and incubated for 3 h at room temperature. The plate was washed 4 times with 400 μl/well wash buffer supplied with the kit. PAPP-A conjugate (100 μl/well) was added and incubated for 2 h at room temperature. The plate was washed 4 times with 400 μl/well wash buffer before Substrate solution (100 μl/well) was added for 30 min at room temperature while protecting the plate from the light. Stop solution (100 μl/well) was added and a colour change from blue to yellow was observed. The OD<sub>450</sub> was then determined using a Victor X Multilabel Plate reader (Perkin-Elmer, Oy, Finland). A standard curve was plotted (A<sub>450</sub> vs. concentration) and used to determine the PAPP-A concentration of the unknown samples (appendix 12).

2.10. Measurement of cell proliferation
2.10.1 BrdU proliferation assay
A bromodeoxyuridine (BrdU) cell proliferation ELISA, which is a non-radioactive measurement of DNA synthesis (Roche Applied Sciences, Basel, SWI) based on the incorporation of BrdU into DNA was used to determine PC-3M-luc2 cell growth. Briefly, BrdU labelling reagent (20μl/well) was added to cells in a 96 well plate and incubated for 24 h. The BrdU labelling reagent was removed and FixDenat (200μl/well) was added for 30 min at room temperature to fix the cells in the plate. The FixDenat solution was removed, anti-BrdU-POD working solution was added (100μl/well) and the plate was then incubated for 90 min at room temperature. The antibody conjugate was removed and the wells were rinsed 3 times with wash buffer (300μl/well). Substrate solution was added to each well (100μl/well) and incubated until the colour development was sufficient for photometric detection (25 min). The absorbance was measured using the Victor X Multilabel plate reader (Perkin-Elmer, Oy, Finland) set to 490nm. The blank measurement was subtracted from each reading and the increase of cell proliferation was seen as the percentage increase in proliferation relative to the untreated controls, which were taken as 100 % proliferation.
2.10.2 MTS Assay
The CellTiter 96® AQ™ Non-Radioactive Cell Proliferation Assay (Promega, WI, USA) is a colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The CellTiter 96® AQ™ Assay is composed of solutions of a tetrazolium compound \([3-(4,5\text{-dimethylthiazol-2-yl})-5-(3\text{-carboxymethoxyphenyl})-2-(4-sulfophenyl)]\text{-}2H\text{-tetrazolium, inner salt, MTS}\) and an electron coupling reagent (phenazine methosulfate) PMS. MTS is reduced by cells into a formazan product that is soluble in tissue culture medium. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product is measured at 490 nm, which is directly proportional to the number of living cells in culture. MTS was added to cells in a 96 well plate (20 µl/100 µl medium) and incubated for 1.5 h at 37°C. The absorbance was measured using the Victor X Multilabel plate reader (Perkin-Elmer, Oy, Finland) set to 490nm. The blank measurement was subtracted from each reading, controls (untreated) were taken as 100 % proliferation and an increase or decrease in proliferation was relative to untreated controls.

2.11 In vivo experiments
2.11.1. Animals
Animal experiments were carried out for 2 months in Dana Farber Cancer Institute, Boston, MA. 10-12 week old male NSG (NOD SCID Beige gamma) mice were used for all experiments. NSG mice were obtained from a breeding colony in Charles River (Charles River Laboratories, MA) and were housed in a licensed biomedical facility (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA) and had ad libitum access to animal chow, water and 12 h light 12 h darkness cycle. All procedures were subjected to institutional ethics review carried out under the Animal licence and guidelines from the Institutional Animal Care and Use Committee (IACUC) and in accordance with the USA Animal Welfare Act.

2.11.2 Preparation of cells for injection
PC-3M-luc2 dBP4 cells were grown to 80 % confluency in 20 cm² dishes. Cells were washed with 5 ml of PBS pre-warmed to 37 °C. 3 ml of pre-warmed trypsin-EDTA was added to the cells and incubated at 37 °C until cells detached. 20 ml MEM Eagle containing 10 % (v/v) FCS was added to neutralise the trypsin. Cells were pelleted at 1100 x g for 3 min. Supernatant was removed and cells were resuspended in 5 ml MEM Eagle containing 10 % (v/v) FCS. Cells were counted (section 2.2.5), pelleted at 1100 x g for 3 min and resuspended in the appropriate volume of PBS (without Mg²⁺/Ca²⁺) to the correct concentration of cells for each animal model.
2.11.3 Subcutaneous prostate cancer model

10-12 week old male NSG mice were anaesthetised in an anaesthetic chamber with 2 % (v/v) isoflurane using 1 L/min oxygen. Animals were placed on their side with an anaesthetic gas pipe covering their noses and mouths and anaesthesia was maintained at 2 % (v/v) isoflurane (Baxter, IL, USA) using 1 L/min oxygen. PC-3M-luc2 dBP4 cells were suspended in a 70 % (v/v) PBS: 30 % (v/v) Matrigel solution. Cells were kept on ice throughout the procedure in order to maintain the Matrigel (BD Biosciences, NJ, USA) in a liquid form. Cells were drawn directly into a 1 cc BD syringe before attaching the 27 gauge needle to avoid shearing. The barrel was tapped to remove any air bubbles and the cell suspension was pushed through to remove all air present. An alcohol prep pad was used to disinfect the injection site. The needle was inserted subcutaneously near the top of the right leg muscle and gently moved from side-to-side to create a pocket for the cells to grow in. 100 μl (5 × 10⁶ PC-3M-luc2 dBP4 cells) of the cell suspension was injected into the pocket created.

2.11.3.1 Measurement of subcutaneous tumours

Following implantation of PC-3M-luc2 dBP4 subcutaneously in male NSG mice, tumour measurements were carried out every 3 - 4 days using a caliper. The length and width of tumours were measured in millimetres (mm). MTD was calculated using the equation V = length × width. Treatment of mice with doxycycline chow (200 mg/kg, Bio-Serve, Laurel, MD) or TKI, NVP-AEW541 (50 mg/kg diluted in tartaric acid, given daily by oral gavage) was started when tumours reached a MTD of 7 - 8 mm (23 days post implantation of PC-3M-luc2 dBP4 cells (treatment day 1)). NSG mice were treated for up to 42 days.

2.11.4 Orthotopic prostate cancer model

10-12 week old male NSG mice were anaesthetised in an anaesthetic chamber with 2 % (v/v) isoflurane (Baxter, IL, USA) using 1 L/min oxygen. Animals were placed on their back with an anaesthetic gas pipe covering their noses and mouths and anaesthesia was maintained at 2 % (v/v) isoflurane using 1 L/min oxygen. Under anaesthesia, a 1.5 cm skin incision was made directly anterior to the abdominal fat pad. Skin was gently dissociated from the underlying peritoneum using a surgical scissors. A similar incision was made in the peritoneum, directly under the original cut. The bladder was pulled through the incision, at which point the dorsal prostate was located and injected with 5 × 10⁵ PC-3M-luc2 dBP4 cells in 50 μl 1:1 PBS: Matrigel solution using a 100 μl 1710 Hamilton syringe with a 27 gauge needle. PBS: Matrigel solution was kept on ice throughout the procedure to maintain the Matrigel in a liquid form. After injections, both incisions were sutured. The peritoneum was sutured using Ethicon 4.0 Chromic Gut (absorbable) sutures and the skin was sutured using Ethicon 4.0 SofSilk sutures. Mice received two analgesics, topical Marcaine (0.5 % (w/v)
bupivacaine, Hospira Inc., TX, USA) directly on the incision site and an i.p. injection (100 μl) of Buprenex (0.3 mg buprenorphine base/ml, Reckitt Benckiser Healthcare, NJ, USA) upon waking from anaesthesia. Mice were monitored daily until the surgical wound fully healed. Treatment with doxycycline chow (200 mg/kg) or TKI, NVP-AEW541 (50 mg/kg diluted in tartaric acid, given daily by oral gavage) was initiated 22 days post implantation of PC-3M-luc2 dBP4 cells (treatment day 1) and mice were treated for 26 days.

2.11.5 Intracardiac metastasis model

Ultrasound guided intracardiac injection of tumour cells was carried out using the Vevo 770 High-Resolution Imaging System (VisualSonics, Toronto, Canada) with a 703 scan head (35 MHz). 10-12 week old NSG mice were anaesthetised in an anaesthetic chamber with 2 % (v/v) isoflurane (Baxter, IL, USA) using 1 L/min oxygen. Animals were placed on their back on a stereotactic board with an anaesthetic gas pipe covering their noses and mouths and anaesthesia was maintained at 2 % (v/v) isoflurane using 1 L/min oxygen. PC-3M-luc2 dBP4 cells were drawn directly into a 1 cc BD syringe before attaching the 27 gauge needle to avoid shearing. The barrel was tapped to remove any air bubbles and the cell suspension was pushed through to remove all air present. The syringe was placed into a stereotactic device and orientated toward the heart. Ultrasound was used to locate the left ventricle. The needle was inserted between the ribs, once the ultrasound detected that the needle was at the left ventricle 100 μl of 5 x 10^4 PC-3M-luc2 dBP4 cells were injected. Doxycycline (200 mg/kg) treatment was initiated 42 days post injection of PC-3M-luc2 dBP4 cells and mice were treated for up to 20 days.

2.11.6 Intravenous metastasis model

10-12 week old male NSG mice were injected via the lateral tail vein. PC-3M-luc2 dBP4 cells were drawn directly into a 1 cc BD syringe before attaching the 27 gauge needle to avoid shearing. The barrel was tapped to remove any air bubbles and the cell suspension was pushed through to remove all air present. Before injection each mouse was vasodilated by placing under a warming lamp for 1-2 min to increase blood flow through the area, before being placed in a tail vein restrainer (Braintree Scientific, MA). Prior to injection the entire tail vein was disinfected with an alcohol prep pad. 2 x 10^6 PC-3M-luc2 dBP4 cells in 250 μl PBS were injected into NSG mice via the lateral tail vein. After the needle was removed from the vein a 2 x 2 cm gauze pad was used to staunch bleeding from the injection site. Doxycycline (200 mg/kg) treatment was initiated 47 days post injection of PC-3M-luc2 dBP4 cells and mice were treated for up to 32 days.

59
2.11.7 Bioluminescent imaging
The IVIS Spectrum (Caliper Life Sciences, MA, USA) was used to image mice injected with PC-3M-luc2 dBP4 cells. The PC-3M-luc2 dBP4 cells express luciferase so upon injection of luciferin light is emitted which is detected using the IVIS Spectrum. 250 μl of d-luciferin (75 mg/kg, Caliper Life Sciences, MA, USA) was administered to mice by i.p. injection and mice were imaged 5 min later under 2% (v/v) isoflurane (Baxter, IL, USA) using 1 L/min oxygen. Bioluminescence was measured for 1 s at f/stop 1. Images were then analysed using Living Image 3.2 software. To allow direct comparison of images and quantification, images were recorded as p/s/cm²/sr (total photons per centimetre squared per steradian).

2.11.8 Blood Glucose measurement
Blood glucose levels were measured in mice using an AlphaTRAK glucose meter (Abbott, IL, USA). Before measuring blood glucose, a control solution was used to validate the test. A new test strip was inserted and 2-3 drops of blood were placed on the strip and glucose levels were read as mmol/L.

2.11.9 Removal of tumours
NSG mice were euthanised in a chamber with 100 % CO₂. Subcutaneous and prostate tumours were excised. For protein analysis each tumour was placed into a 1.5 ml tube and snap frozen in liquid nitrogen, then stored at -80°C. For the intracardiac and IV metastasis models mice were assessed for the presence of metastases in the abdominal cavity and the whole mouse was placed into 10 % (v/v) buffered formalin for 48 h. After 48 h fixed mice were transferred to 70 % (v/v) EtOH.

2.11.10 Serum collection
Blood was collected promptly after CO₂ asphyxiation via cardiac puncture and placed into 1.5 ml tube. The blood was mixed by gentle inversion and serum was separated by centrifugation at 3000 x g for 10 min at room temperature. Serum was then recovered and stored at -80°C.

2.12 Statistical Analysis
For statistical analysis of IGF IR staining in prostate TMAs, tumours were scored as low expression (0/1) (cores with negative or weak staining) or high expression (2/3), (cores with moderate and strong staining). Fisher’s Exact tests were performed on contingency tables using Graphpad INSTAT-3™ (Graphpad Software Inc, San Diego, USA) to analyse associations between prostate cancer samples, BPH/normal samples or Gleason grade and immunohistochemical score (negative (0/1) and positive (2/3)).
Where means for more than two groups were compared, statistical comparison was carried out by One-Way ANOVA with Dunnett post hoc test to examine differences from the controls. To carry out multiple comparisons, LSD post hoc correction was used. Where the means of two data sets were compared an unpaired Student t-test was used. To compare two dependent data sets a paired t-test was used. To determine correlations between two groups, a Pearson’s correlation was carried out. A Kaplan-Meier curve was used in survival curves. Statistics was carried out using SPSS™ statistical package.
Chapter 3
Involvement of the IGF pathway in prostate cancer
3.1 Introduction

PSA measurement is the most commonly used diagnostic tool for prostate cancer detection, as PSA is elevated in prostate cancer (Fall et al., 2007). PSA velocity (doubling time) is also used in prostate cancer diagnosis and screening (Carter et al., 2006). However, current opinion regarding the value of PSA velocity as a diagnostic tool is conflicting. It has been reported that PSA velocity is a significant independent clinical factor predicting relapse after radical prostatectomy and also predicts for larger, more aggressive and more locally advanced tumours (Patel et al., 2005). In contrast to Patel et al (2005), no evidence to support the recommendation that men with high PSA velocity should be biopsied in the absence of other indications such as elevated PSA or positive digital rectal exam (DRE) was seen. This study showed that there is little evidence that PSA velocity adds to the predictive value of PSA alone (Vickers et al., 2011). Therefore, a new panel of biomarkers for prostate cancer are needed to enhance the sensitivity for prostate cancer detection. Upon diagnosis of biopsy-confirmed prostate cancer the tumour is assigned a stage and grade. Staging of prostate cancer gives information on the extent of the cancer and whether it has spread to other organs. The TNM (tumour, node, metastasis) staging system (described in TNM atlas) describes the extent of the primary tumour (T), the absence or presence of metastasis to nearby lymph nodes or glands (N) and the absence or presence of distant metastasis (M) (Wittekind et al (2005)). Stage T1 tumours are clinically unapparent and are not detected by DRE, stage T2 tumours are confined within the prostate, stage T3 tumours spread beyond the outer layer of the prostate on one or both sides and may have spread to the seminal vesicles and stage T4 tumours have spread beyond the seminal vesicles to nearby tissue or organs, such as the rectum, bladder, or pelvic wall (Shambaugh et al, 2003)). Staging can help in determining the optimum course of treatment required for each individual patient. The Gleason grade gives information on the tumour's behaviour. Gleason grade is numbered 1 - 5, a tumour with a low-grade (1) is likely to be slow-growing while one with a high-grade (5) is more likely to be aggressive or have spread outside the prostate. The Gleason score is the sum of the two most common Gleason grade patterns in the prostate tissue. The Gleason score can range from 2 - 10. A score of 2 - 4 is considered low-grade, a score of 5 - 7 an intermediate grade and 8 - 10, high-grade (Understanding Gleason grade (1997)).

The main risk factors for prostate cancer development are age, race, family history and diet (Giovannucci et al., 2007). The exact mechanism of prostate cancer development is complex as numerous signalling pathways are involved. One such pathway is the IGF pathway. Over the last number of years interest has grown in targeting the IGF pathway in prostate cancer. Stattin et al (2000) showed a statistically significant association between prostate cancer risk and increasing levels of circulating IGF I (odds ratio 1.57) particularly in
younger men suggesting the involvement of IGF I in early stages of prostate cancer. Others have shown that the IGF IR is overexpressed in prostate cancer and commonly persists in metastatic disease (Hellawell et al., 2002) further supporting the involvement of the IGF pathway in the pathogenesis of the disease. The PI3K/Akt and MAPK pathways are IGF IR main downstream targets. IGF I can inhibit apoptosis via activation of the PI3K or MAPK pathways (Párrizas et al., 1997b). Akt is a serine threonine kinase downstream of PI3K that regulates apoptosis and proliferation (Kim et al., 2001, Rössig et al., 2001). High levels of activated Akt (phospho Akt) in prostate cancer tissue are a strong predictor of elevated PSA post curative therapy (Ayala et al., 2004). Immunohistochemical analysis of prostate cancer tissue using antibodies to phospho Akt and phospho MAPK showed an increase in activated Akt in high Gleason grade tumours, whereas activated MAPK was decreased relative to PIN (Malik et al., 2002). This may imply that the PI3K pathway is the dominant pathway involved in prostate cancer progression. As well as changes in IGF I, IGF IR and downstream effectors, IGFBPs are known to be increased or decreased in prostate cancer relative to healthy individuals.

Circulating IGFBP 3 is the most abundant of the IGF binding proteins (Baxter et al., 1989) is decreased in prostate cancer. This decrease in IGFBP 3 in combination with an increase in plasma IGF I is associated with advanced stage prostate cancer (Chan et al., 2002). IGFBP 2 is the second most abundant binding protein in the body (Wheatcroft and Kearney, 2009). Elevated serum IGFBP 2 levels have been noted in prostate cancer patients and are associated with PSA levels and tumour stage (Cohen et al., 1993). IGFBP 4 mRNA and immunostaining have shown IGFBP 4 to be elevated within epithelial cells of prostate adenocarcinoma relative to benign tissue (Tennant et al., 1996c). In vivo, overexpression of IGFBP 4 in androgen independent M12 prostate cancer cells implanted subcutaneously in male nude athymic mice markedly delayed tumour growth (Damon et al., 1998). This suggests that increased IGFBP 4 may have an inhibitory role in prostate cancer tissue. However, PSA levels are elevated in the majority of prostate cancer patients and PSA cleaves IGFBP 4 (Rehault et al., 2001). Therefore, an increase in IGFBP 4 and PSA could trigger proteolysis of IGFBP 4 thus leading to increased IGF I to stimulate tumour growth. A protease resistant IGFBP 4 may be useful in tumour growth inhibition as it could not be cleaved by proteases, inhibiting IGF I proliferative effects.

As the major clinical challenge in prostate cancer patients is treatment of androgen independent disease an androgen independent cell line (PC-3M-luc2) was chosen for this in vitro and in vivo study. An in vivo model (SCID mice) of progression from androgen dependent to androgen independence using LAPC-9 and LNCaP prostate cancer cells has shown an increase in IGF I (60-fold and 28-fold, respectively) and IGF IR mRNA (2.5-fold and 5-fold, respectively) in the androgen independent model relative to androgen dependent
neoplasms, suggesting IGF IR signalling may confer androgen independence (Nickerson et al., 2001). Increased Akt activity has been seen in an androgen independent, PSA positive LNAI cell line relative to the parental androgen dependent LNCaP cells (Graff et al., 2000). Literature suggests that the IGF pathway plays a role in the pathogenesis of prostate cancer and may provide a therapeutic target for this disease.

The main aims of this chapter were to assess the expression of the IGF IR in prostate cancer tissue and to determine the expression of components of the IGF pathway in an androgen independent prostate cancer cell line, PC-3M-luc2. In addition, to determine the suitability of these cells to assess the therapeutic potential of a protease resistant IGFBP 4 in an in vivo model of androgen independent prostate cancer.

**The specific aims of this chapter were:**

1. To investigate the expression of the IGF IR in prostate cancer tissue compared to normal tissue and to assess a correlation between IGF IR expression and Gleason grade.
2. To determine the expression of IGF pathway components in the human prostate cancer cell line PC-3M-luc2 in order to assess their suitability for use in vivo.
3. To examine whether the IGF IR interacts with IR and EGFR in PC-3M-luc2 cells.
4. To assess downstream activation of the IGF pathway following IGF stimulation in PC-3M-luc2.
3.2 Results

3.2.1 IGF IR expression in prostate cancer TMAs

Tumour tissue was obtained from 45 prostate cancer patients with Gleason score ranging from 6 - 9 (average number of cores/patient = 7). The clinicopathological details of these patients are detailed in Table 3.1. Gleason grade is based on microscopic tumour patterns giving information on the tumour’s behaviour. The Gleason score is the sum of the two most common Gleason grade patterns in the prostate tumour. Gleason score is based on the degree of loss of normal glandular structure (de-differentiation). Figure 3.1 shows representative cores for benign prostatic hyperplasia (BPH) and Gleason grade 3 - 5. BPH tissue shows prostatic hyperplasia consisting of nodules of glands and intervening stroma, the glands vary in size with larger glands showing greater papillary folding (Figure 3.1 A). Gleason grade 3 tissue is well differentiated with normal gland unit similar to the normal prostate and glands vary in shape and invade into stroma (Figure 3.1 B). Gleason grade 4 shows disruption and loss of normal gland structure (Figure 3.1 C). Gleason grade 5 is undifferentiated with no evidence of gland structure (Figure 3.1 D). Expression of the IGF IRβ subunit was assessed in the prostate cancer TMAs (n=263 cores). Immunoreactivity was seen predominantly in epithelial cells surrounding the prostate glands and assigned a score of 0 - 3 based on intensity of staining. Figure 3.2 shows representative sections for each of the immunoreactivity scores numbered 0 - 3. A representative Gleason grade 4 core shows negative IGF IRβ staining (0, Figure 3.2 A), a representative Gleason grade 4 core shows weak IGF IRβ staining (+ 1, Figure 3.2 B). A representative BPH core shows moderate IGF IRβ staining (+ 2, Figure 3.2 C) and a representative Gleason grade 3 shows strong IGF IRβ staining (+ 3, Figure 3.2 D).

Membrane and cytoplasmic staining was noted in prostate cancer tissue. Membrane and cytoplasmic staining were scored separately using the scoring system represented in Figure 3.2. Representative sections are shown (Figure 3.3) for membrane or cytoplasmic staining. In Figure 3.3 A, BPH IGF IRβ membrane staining was predominantly around the membrane with no IGF IRβ cytoplasmic staining, while cytoplasmic staining for IGF IRβ was weak, but diffuse in the cytoplasm with no IGF IRβ membrane staining. In Gleason grade 3, IGF IRβ membrane staining was strong with moderate cytoplasmic staining, while cytoplasmic staining was weak with no evidence of membrane IGF IRβ staining (Figure 3.3 B). In Gleason grade 4, membrane staining showed strong membrane IGF IRβ staining with weak cytoplasmic staining, while cytoplasmic staining showed moderate IGF IRβ staining in cytoplasm and negative for IGF IRβ membrane staining (Figure 3.3 C) and in Gleason grade 5 membrane staining showed IGF IRβ was weak with
Table 3.1: Clinicopathological parameters of patients for TMA analysis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>45</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>58</td>
</tr>
<tr>
<td>Range</td>
<td>42 - 70</td>
</tr>
<tr>
<td><strong>PSA levels (ng/ml)</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>8.6</td>
</tr>
<tr>
<td>Range</td>
<td>4.4 - 19</td>
</tr>
<tr>
<td><strong>Gleason grade score</strong></td>
<td></td>
</tr>
<tr>
<td>sum (n)</td>
<td></td>
</tr>
<tr>
<td>Gleason score 6</td>
<td>25</td>
</tr>
<tr>
<td>Gleason score 7</td>
<td>17</td>
</tr>
<tr>
<td>Gleason score 8</td>
<td>2</td>
</tr>
<tr>
<td>Gleason score 9</td>
<td>1</td>
</tr>
<tr>
<td><strong>Tumour Stage (n)</strong></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>34</td>
</tr>
<tr>
<td>T3</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 3.1: Haematoxylin and Eosin (H&E) staining of prostate tissue. Prostate cancer tissue microarrays were stained with H&E (n=263 cores) to determine Gleason grade. Representative sections of (A) Benign Prostatic Hyperplasia (BPH). (B) Gleason grade 3, (C) Gleason grade 4 and (D) Gleason grade 5. (Original magnification 200 ×).
Figure 3.2: IGF IRβ expression in prostate cancer TMAs. TMAs (n=263 cores) were stained with an anti-IGF IRβ antibody. Representative sections illustrating the IGF IRβ scoring (0-3). (A) 0, Gleason grade 4 core with no IGF IRβ staining, (B) +1, Gleason grade 4 core with weak IGF IRβ staining, (C) +2, a BPH core with moderate IGF IRβ staining and (D) +3, Gleason grade 3 core with strong IGF IRβ staining. (Original magnification 200 x).
weak cytoplasmic staining, while cytoplasmic staining showed weak cytoplasmic IGF IRβ with no IGF IRβ membrane staining (Figure 3.3 D). Membrane IGF IRβ varied from weak-strong staining whereas IGF IRβ cytoplasmic staining only varied from weak-moderate. Membrane IGF IRβ staining was more prominent than cytoplasmic IGF IRβ staining.

In order to establish the relationship between IGF IRβ staining and Gleason grade in the prostate cancer patient TMAs, statistical analysis was carried out for either membrane or cytoplasmic staining. Tumour cores were categorised into negative (0/1) or positive (2/3) based on staining intensity. Table 3.2 shows the association between membrane IGF IRβ staining and Gleason grade as determined by Fisher's Exact Test. A p value <0.05 was taken as significant. Membrane IGF IRβ expression was increased in tumour tissue relative to normal/BPH tissue (p<0.001). Membrane IGF IRβ expression was higher in Gleason grade 3 (p<0.0004) and Gleason grade 4 (p<0.002) relative to normal tissue. Membrane IGF IRβ staining was higher in Gleason grade 3 than Gleason grade 5 (p<0.005); there was no significant difference in membrane IGF IRβ expression between BPH/normal tissue and Gleason grade 5 tissue.

Table 3.3 shows the association between cytoplasmic IGF IRβ staining and Gleason grade as determined by Fisher's Exact Test. A p value <0.05 was taken as significant. There was no significant difference in cytoplasmic IGF IRβ between normal/BPH tissue and overall tumour tissue. Cytoplasmic IGF IRβ expression increased in Gleason grade 3 relative to normal/BPH tissue (p<0.02). Gleason grade 3 expressed higher levels of cytoplasmic IGF IRβ compared to Gleason grade 5 (p<0.02), but there was no significant difference in cytoplasmic IGF IRβ expression between BPH/normal tissue and Gleason grade 4 or 5 tissue. A Pearson's correlation was carried out to assess if there was an association between membrane IGF IRβ and cytoplasmic IGF IRβ staining. A value close to 1 represented a positive correlation and values close to -1 represented a negative correlation. A value of p<0.05 was taken as significant. A positive correlation was noted between membrane IGF IRβ and cytoplasmic IGF IRβ (p<0.01) (Table 3.4). Therefore, as membrane IGF IRβ staining increased an increase in cytoplasmic IGF IRβ was also seen.

3.2.2 Plasma IGF I and PAPP-A levels in BPH and prostate cancer patients
An IGF I ELISA was carried out on plasma samples (n = 33) obtained from the Prostate Cancer Research Consortium. After determining the IGF I levels from each sample, clinical information was obtained. Patient groups were categorised into BPH (n = 13), Gleason score 5 (n = 11) and Gleason score 7 (n = 9). Table 3.5 shows IGF I levels measured for each group. BPH patients were used as controls. A one-way ANOVA was used to compare IGF I levels between each group. No statistically significant difference was found in IGF I.
Figure 3.3: Total IGF1Rβ staining in the membrane and cytoplasm of prostate cancer tissue. Prostate cancer TMAs (n=263 cores) were stained with an anti-IGF IRβ antibody. Membrane and cytoplasmic staining were scored separately using the 0 - 3 scheme illustrated in Figure 3.2. Representative images showing membrane and cytoplasmic staining are shown. (A), BPH tissue shows strong membrane IGF IRβ and weak cytoplasmic staining. (B), Gleason grade 3 tissue shows strong membrane IGF IRβ and weak cytoplasmic IGF IRβ staining. (C) Gleason grade 4 tissue shows strong membrane IGF IRβ and moderate cytoplasmic IGF IRβ and (D) Gleason grade 5 tissue shows weak membrane IGF IRβ and weak cytoplasmic IGF IRβ staining. (Original magnification 200 ×).
Table 3.2: Relationship between IGFIRβ membrane staining and Gleason grade in prostate cancer TMAs (0/1 = Negative, 2/3 = Positive). Tumour cores (n=263) were categorised as negative (0/1) or positive (2/3) based on staining intensity. The association between immunohistochemical score of the IGF IRβ membrane staining and Gleason grade was determined by the Fisher’s Exact Test. A p value of < 0.05 was taken as significant. (ns = non significant).

<table>
<thead>
<tr>
<th>TEST</th>
<th>Histology</th>
<th>Score of Membrane</th>
<th>Score of membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total IGF IR (0/1)</td>
<td>Total IGF IR (2/3)</td>
</tr>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>99</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Tumour n = 115 (40 %) (6 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p &lt; 0.001</td>
<td>Tumour n = 138 (37 %) (17 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason grade 3 vs. Gleason grade 5</td>
<td>42</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>p &lt; 0.005</td>
<td>Gleason grade 5 n = 67 (46 %) (27 %)</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>99</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Gleason grade 3 n = 115 (54 %) (9 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p &lt; 0.0004</td>
<td>Gleason grade 3 n = 67 (23 %) (14 %)</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>99</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Gleason grade 4 n = 115 (61 %) (10 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p &lt; 0.002</td>
<td>Gleason grade 4 n = 46 (18 %) (11 %)</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>99</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Gleason grade 5 n = 115 (7 %) (11 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ns</td>
<td>Gleason grade 5 n = 25 (16 %) (14 %)</td>
<td>23</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 3.3: Relationship between IGF1Rβ cytoplasmic staining and Gleason grade in prostate cancer TMA. (0/1 = Negative, 2/3 = Positive). Tumour cores (n=263) were categorised as negative (0/1) or positive (2/3) based on staining intensity. The association between immunohistochemical score of the IGF IRβ cytoplasmic staining and Gleason grade was determined by the Fisher’s Exact Test. A p value of < 0.05 was taken as significant. (ns = non significant).

<table>
<thead>
<tr>
<th>TEST</th>
<th>Histology</th>
<th>Score of cytoplasmic Total IGF IR (0/1)</th>
<th>Score of cytoplasmic Total IGF IR (2/3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>96 (38 %)</td>
<td>19 (8 %)</td>
<td></td>
</tr>
<tr>
<td>Tumour ns</td>
<td>102 (40 %)</td>
<td>36 (14 %)</td>
<td></td>
</tr>
<tr>
<td>Gleason grade 3 vs. Gleason grade 5</td>
<td>46 (50 %)</td>
<td>21 (23 %)</td>
<td></td>
</tr>
<tr>
<td>p&lt; 0.02</td>
<td>23 (25 %)</td>
<td>2 (2 %)</td>
<td></td>
</tr>
<tr>
<td>n = 67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>96 (53 %)</td>
<td>19 (10 %)</td>
<td></td>
</tr>
<tr>
<td>Gleason grade 3</td>
<td>46 (25 %)</td>
<td>21 (12 %)</td>
<td></td>
</tr>
<tr>
<td>p&lt; 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>96 (60 %)</td>
<td>19 (12 %)</td>
<td></td>
</tr>
<tr>
<td>Gleason grade 4</td>
<td>33 (20 %)</td>
<td>13 (8 %)</td>
<td></td>
</tr>
<tr>
<td>ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal vs. Normal/BPH</td>
<td>96 (69 %)</td>
<td>19 (14 %)</td>
<td></td>
</tr>
<tr>
<td>Gleason grade 5</td>
<td>23 (16 %)</td>
<td>2 (1 %)</td>
<td></td>
</tr>
<tr>
<td>ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4 Pearson's correlation coefficients between membrane IGF IRβ and cytoplasmic IGF IRβ staining. Pearson's correlation was used to analyse the association of membrane IGF IRβ with cytoplasmic IGF IRβ staining. **p<0.01.

<table>
<thead>
<tr>
<th>Membrane IGF IRβ staining</th>
<th>Cytoplasmic IGF IRβ staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>0.366**</td>
</tr>
<tr>
<td>IGF IRβ staining</td>
<td>0.366**</td>
</tr>
<tr>
<td>Cytoplasmic IGF IRβ staining</td>
<td></td>
</tr>
<tr>
<td>IGF IRβ staining</td>
<td>-</td>
</tr>
</tbody>
</table>
levels between each group. A PAPP-A ELISA was carried out on the same plasma samples used for the IGF I ELISA. BPH patients were used as controls. Table 3.6 shows PAPP-A levels measured for each group. One-way ANOVA was used to compare PAPP-A levels between groups. A value of $p<0.05$ was considered as significant. Negative values were obtained for BPH and Gleason score 7 patients therefore a value of zero was given. This may be due to a lack of sensitivity with the PAPP-A ELISA assay. PAPP-A was significantly higher in Gleason score 5 patients relative to controls ($p<0.0001$ vs. BPH).

Pearson's correlation was performed to determine whether there was a relationship between IGF I, PAPP-A, PSA levels, age and Gleason score (Table 3.7). A negative correlation was seen between PAPP-A levels and age ($p<0.01$). PAPP-A decreased with age. There was no correlation seen between PAPP-A and IGF I, PAPP-A and PSA, or PAPP-A and Gleason score. No correlation was seen between IGF I and PSA, age or Gleason score.

### 3.2.3 Expression of the IGF pathway in PC-3M-luc2 cells

IGF IR expression was assessed by immunoblotting for the β subunit of the IGF IR. PC-3M-luc 2 and MCF-7 whole cell lysate was fractionated by 4 - 20 % (w/v) SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with anti-IGF IRβ antibody (Figure 3.4). The predicted molecular weight of IGF IRβ is 95 kDa. IGF IRβ was expressed by MCF-7 (positive control, lane 1) and PC-3M-luc2 cells (lane 2).

IGFBPs and IGF I/II expression were determined using a multiplex RT-PCR, which allows multiple binding proteins to be assessed within one sample at the same time. PCR products were fractionated on a 2 % (w/v) agarose gel (Figure 3.5). Amplification of the 18S ribosomal subunit was a control for loading and sample integrity. PC-3M-luc2 cells express IGFBP 3 (lane 3). To further validate IGFBP 3 expression in PC-3M-luc2 cells, Western blot analysis was carried out. After culturing in serum-free medium for 48 h, PC-3M-luc2 conditioned medium was collected and concentrated using 3 kDa Centricon YM-3 filter tube. PC-3M-luc2 conditioned medium and whole cell lysate along with MIA PaCa2 cell lysate (pancreatic cell line), which are known to express IGFBP 3, were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-IGFBP 3 antibody (Figure 3.6 A). Predicted size of IGFBP 3 is 30 kDa. IGFBP 3 was expressed in PC-3M-luc2 cell lysate (lane 1) and conditioned medium (lane 2). Cell lysate from MIA PaCa2 cells used as a positive control is shown in lane 3.
**Table 3.5: Plasma IGF I in BPH and prostate cancer patients.** Plasma levels of IGF I were measured in duplicate by ELISA in BPH and prostate cancer patients. IGF I levels were compared between the groups using one-way ANOVA (SEM = standard error of the mean) (p = ns).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (Mean ± SEM)</th>
<th>IGF I (ng/ml) (Mean ± SEM)</th>
<th>Pre-op PSA (ng/ml) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH (n = 13)</td>
<td>71 ± 2.2</td>
<td>67.2 ± 5.5</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>Gleason score 5</td>
<td>59 ± 1.3</td>
<td>52.8 ± 5.8</td>
<td>7.9 ± 1.6</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason score 7</td>
<td>62 ± 1.5</td>
<td>70.7 ± 8.7</td>
<td>10.8 ± 1.8</td>
</tr>
<tr>
<td>(n = 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.6: Plasma PAPP-A in prostate cancer patients. Plasma levels of PAPP-A were measured in duplicate by ELISA in BPH and prostate cancer patients. PAPP-A levels were compared between the groups using one-way ANOVA followed by Dunnet post hoc procedure. ***p<0.001 vs BPH. (SEM = standard error of the mean).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (Mean ± SEM)</th>
<th>PAPP-A (ng/ml) (Mean ± SEM)</th>
<th>Pre-op PSA (ng/ml) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH (n=13)</td>
<td>71 ± 2.2</td>
<td>0</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>Gleason score 5</td>
<td>59 ± 1.3</td>
<td>19.4 ± 10.5 ***</td>
<td>7.9 ± 1.6</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason score 7</td>
<td>62 ± 1.5</td>
<td>0</td>
<td>10.8 ± 1.8</td>
</tr>
<tr>
<td>(n = 9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.7 Pearson’s correlation coefficients between measured IGF I and PAPP-A levels. Pearson’s correlation was used to analyse the association of IGF I and PAPP-A with PSA, age and Gleason score. **p<0.01.

<table>
<thead>
<tr>
<th></th>
<th>IGF I</th>
<th>PAPP-A</th>
<th>Pre-op PSA</th>
<th>Age</th>
<th>Gleason score (5 &amp; 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF I</td>
<td>-</td>
<td>-0.092</td>
<td>-0.148</td>
<td>0.075</td>
<td>0.385</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>-0.092</td>
<td>-</td>
<td>0.186</td>
<td>-0.599**</td>
<td>-0.314</td>
</tr>
<tr>
<td>Pre-op</td>
<td></td>
<td>0.186</td>
<td>-</td>
<td>0.097</td>
<td>0.266</td>
</tr>
<tr>
<td>PSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>-0.599**</td>
<td>0.097</td>
<td></td>
<td>0.398</td>
</tr>
<tr>
<td></td>
<td>0.385</td>
<td>-0.314</td>
<td>0.266</td>
<td>0.398</td>
<td></td>
</tr>
</tbody>
</table>

(5 & 7)
Figure 3.4: IGFR β is expressed by PC-3M-luc2 cells. Cell lysate was fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and probed with anti-IGF IRβ. Lane 1 shows cell lysate from MCF-7 cells used as a positive control. Lane 2 shows cell lysate from PC-3M-luc2 cells. Position of molecular weight marker is indicated (kDa). Western blot is representative of three independent experiments.
Figure 3.5: PC-3M-luc2 cells express IGFBP 3 mRNA. Expression of IGFBPs and IGFs was determined by multiplex RT-PCR. Amplification of the 18S ribosomal subunit was used as a control for equal loading and sample integrity. PCR products were fractionated on a 2% (w/v) agarose gel. Lane 1 shows 100 bp DNA ladder, lane 2 shows PCR products from the positive control (provided with the kit) and lane 3 shows PCR products from PC-3M-luc2 mRNA. Amplification of PCR products in the positive control is indicated. Position of molecular weight marker is shown (bp).

<table>
<thead>
<tr>
<th>bp</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>700</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>18S (554 bp)</td>
<td>IGFBP6 (409 bp)</td>
<td>IGFBP5 (337 bp)</td>
</tr>
<tr>
<td></td>
<td>(IGFBP3 (284 bp))</td>
<td>(IGFBP2 (256 bp))</td>
<td>(IGF II (231 bp))</td>
</tr>
<tr>
<td></td>
<td>(IGFBP1 (201 bp))</td>
<td>(IGF I (184 bp))</td>
<td></td>
</tr>
</tbody>
</table>
IGFBP 4 was not included in the multiplex RT-PCR kit; therefore Western blot analysis was performed to assess its expression in PC-3M-luc2 cells. PC-3M-luc2 conditioned medium, whole cell lysate and recombinant human IGFBP 4 were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and then probed for IGFBP 4 expression with anti-IGFBP 4 antibody (Figure 3.6 B). Predicted molecular weight of IGFBP 4 is 28 kDa. PC-3M-luc2 conditioned medium expressed IGFBP 4 (lane 2) with a faint band present in the cell lysate (lane 1). The majority of IGFBP 4 was secreted into the conditioned medium. A size difference was seen between IGFBP 4 in conditioned medium and cell lysate. This difference is due to cleavage of the signal peptide upon IGFBP 4 secretion. Therefore, IGFBP 4 in conditioned medium is smaller in molecular weight compared with IGFBP 4 in cell lysate. Recombinant IGFBP 4 was used as a positive control (lane 3) (Figure 3.6 B).

PAPP-A was assessed by RT-PCR. PCR products were fractionated on a 2 % (w/v) agarose gel (Figure 3.7). cDNA from HEK 293 cells were used as a positive control for PAPP-A mRNA expression (lane 2). β-actin was used as a control for equal loading and sample integrity (lane 4 & 5). PC-3M-luc2 cells express PAPP-A (lane 3).

3.2.4 Cell proliferation and VEGF expression in response to IGF I

As PC-3M-luc2 cells express the IGF IR, cell proliferation in response to IGF I ligand was measured by BrdU assay (Figure 3.8). PC-3M-luc2 cells (3 x 10^3 cells/well) were treated in triplicate with 0-100 ng/ml IGF I for 48 h. As PC-3M-luc2 cells express IGFBP 3 and IGFBP 4 (Figure 3.6) cells were also treated with IGF (E3R) (0 -100 ng/ml) for 48 h. IGF (E3R) is a 70 amino acid IGF I analogue with reduced affinity for IGFBPs. Control cells were treated with the vehicle concentration PBS containing 0.1 % (w/v) BSA (IGF I) or PBS containing 10 mM HCl with 0.5 % (w/v) BSA (IGF (E3R)) equivalent to the highest dose of IGF I or IGF (E3R). Proliferation is expressed as % of control where control is taken as 100 %. IGF (E3R) significantly increased cell proliferation at doses of 20 ng/ml (p<0.004), 40 ng/ml (p<0.005) and 60 ng/ml (p<0.026), whereas, IGF I had no effect on cell proliferation (Figure 3.8). As PC-3M-luc2 cells express IGFBP 3 and IGFBP 4, it is possible that these IGFBPs could have blocked IGF I action in this assay.

The effect of IGF I ligand on the expression of the angiogenic factor, VEGF_{165} was assessed by ELISA. PC-3M-luc2 cells (5 x 10^4 cells/well) were treated in triplicate with IGF I or IGF (E3R) (0 - 100 ng/ml) for 48 h. Controls were treated with the vehicle concentration (PBS containing 0.1 % (w/v) BSA (IGF I) or PBS containing 10 mM HCl with 0.5 % (w/v) BSA (IGF (E3R)) equivalent to the highest dose of IGF I or IGF (E3R). Conditioned medium was collected from the cells and VEGF_{165} levels were measured by ELISA. VEGF_{165} was expressed as pg/mg cell protein.
Figure 3.6 A and B: PC-3M-luc2 cells express IGFBP 3 and IGFBP 4 protein. Whole cell lysate or conditioned medium was fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and probed with anti-IGFBP 3 (A) or anti-IGFBP 4 (B) antibodies. In A, lane 1 shows PC-3M-luc2 cell lysate, lane 2 shows conditioned medium from PC-3M-luc2 cells and lane 3 shows MIA PaCa2 cell lysate (pancreatic cell line) which was used as a positive control. In B, lane 1 shows PC-3M-luc2 cell lysate, lane 2 shows conditioned medium from PC-3M-luc2 cells and lane 3 shows recombinant IGFBP 4 as a positive control. Position of molecular weight marker is indicated (kDa). Western blots are representative of three independent experiments.
Figure 3.7: PAPP-A mRNA is expressed by PC-3M-luc2 cells. PAPP-A expression was assessed by RT-PCR. cDNA from HEK 293 cells was used as a positive control for PAPP-A expression. Lane 1 shows 1 kb DNA ladder. Lanes 2 and 3 show PAPP-A mRNA transcript from HEK 293 cells and PC-3M-luc2 cells, respectively. Lanes 4 and 5 show β-actin amplified from HEK 293 cells and PC-3M-luc2 cells, respectively as a control for equal loading and sample integrity. Position of molecular weight marker is indicated (bp). Gel shown is representative of three independent experiments.
Figure 3.8: PC-3M-luc2 cells proliferate in response to IGF (E3R). $3 \times 10^3$ PC-3M-luc2 cells were treated in triplicate with IGF I or IGF (E3R) for 48 h and proliferation was assayed by BrdU incorporation. Proliferation is expressed as % of control where control is taken as 100%. Controls were treated with the vehicle concentration equivalent to the highest dose of IGF I or IGF (E3R). Data ($n = 3$) are expressed as mean ± SEM and analysed by one-way ANOVA with Dunnets post hoc procedure. *p<0.05 vs. Control, **p<0.01 vs. Control.
Treatment with IGF (E3R) had a significant effect on VEGF<sub>165</sub> expression at doses of 60 ng/ml (p<0.017), 80 ng/ml (p<0.035), and 100 ng/ml (p<0.031) whereas IGF I had no statistically significant effect on VEGF<sub>165</sub> production. As PC-3M-luc2 cells express IGFBP 3 and IGFBP 4 this may have inhibited the effects of IGF I (Figure 3.9).

3.2.5 Activation of IGF signalling by IGF I in PC-3M-luc2 cells

Activation of the IGF IR and downstream signalling molecules Akt and MAPK were assessed by Western blotting following IGF I or IGF (E3R) treatment. PC-3M-luc2 cells (1.5 x 10<sup>5</sup> cells/well) were treated with 100 ng/ml IGF I or IGF (E3R) for 0 - 60 min. PC-3M-luc2 whole cell lysates were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-IGF IR<sub>p</sub>, anti-plGF IR (Tyr 1131) and anti-β-actin antibodies (Figure 3.10 A & B). Figure 3.10 A and 3.10 B show no phosphorylation of the IGF IR at zero timepoint. However, following stimulation with IGF I (A) or IGF (E3R) (B) IGF IR activation is seen after 10 min and remains up to 60 min after stimulation. β-actin shows equal loading in all lanes.

To assess expression of Akt, PC-3M-luc2 cells (1.5 x 10<sup>5</sup> cells/well) were treated with 500 nM wortmannin (PI3K inhibitor) for 1 h followed by treatment with 100 ng/ml IGF I (Figure 3.11 A) or IGF (E3R) (Figure 3.11 B) for 0 - 60 min. PC-3M-luc2 whole cell lysates were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-Akt, anti-phospho Akt (Ser 473) and anti-β-actin antibodies (Figure 3.11 A & B). Total Akt is unaffected by wortmannin treatment (Figure 3.11 A & B) and pAkt is absent at the zero timepoint. In cells treated with IGF I Akt is phosphorylated after 10 min of IGF I treatment, but is undetectable after 60 min. PC-3M-luc2 cells treated with IGF (E3R) showed phosphorylation of Akt after 5 min and this was maximal at 15 min. Therefore, IGF I stimulation of Akt at 10 min compared with 5 min in IGF (E3R) stimulated cells may be due to binding of IGF I to IGFBP 3 and IGFBP 4. Wortmannin inhibited the phosphorylation of Akt at all timepoints following stimulation with IGF I or IGF (E3R) (Figure 3.11), suggesting that activation of Akt occurs via a PI3K dependent pathway.

To assess expression and activation of ERK, PC-3M-luc2 cells (1.5 x 10<sup>5</sup> cells/well) were treated with 100 ng/ml IGF I or IGF (E3R) for 0 - 60 min. PC-3M-luc2 whole cell lysates were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-ERK (p44/p42), anti-phospho ERK (Thr202/Tyr204) and anti-α tubulin antibodies. Figure 3.12 A shows PC-3M-luc2 cells stimulated with IGF I had no change in total ERK. There was no difference in pERK at any timepoint.
Figure 3.9: IGF (E3R) stimulates VEGF$_{165}$ production in PC-3M-luc2. PC-3M-luc2 cells were treated with IGF I or IGF (E3R) for 48 h in triplicate and VEGF$_{165}$ was measured by ELISA. Controls were treated with the vehicle concentration equivalent to the highest dose of IGF I or IGF (E3R). Total cell protein was assessed by Bio-Rad DC protein assay. Data (n = 3) are expressed as mean ± SEM and analysed by one-way ANOVA with Dunnet procedure post hoc correction. *p< 0.05 vs. Control.
Figure 3.10 A & B: IGF treatment activates phosphorylation of the IGF IR. 1.5 x 10^5 cells/well PC-3M-luc2 cells were treated with IGF I (100 ng/ml) (A) or IGF (E3R) (100 ng/ml) (B) at different time points (0 - 60 min). 25 μg of cell lysate was fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and then probed for IGF IRβ or pIGF IR (Tyr 1131). β-actin was probed as a loading control. Position of molecular weight marker is indicated (kDa). Western blots are representative of three independent experiments.
**Figure 3.11 A and B: IGF (E3R) induces phosphorylation of Akt mediated by PI3K.**

1.5 x 10^5 cells/well of PC-3M-iuc2 were pre-treated with the PI3K inhibitor, wortmannin (500 nM) for 1 h. Cells with (+) or without (-) wortmannin were stimulated with 100 ng/ml IGF I (A) or IGF (E3R) (B) from 0 - 60 min. Cells were lysed and 25 μg of total cell protein was fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred onto nitrocellulose and probed with anti-Akt, anti-phospho Akt (Ser 473) or β-actin antibody. Position of molecular weight marker is indicated (kDa). Western blots are representative of three independent experiments.
Similarly, PC-3M-luc2 cells treated with IGF (E3R) showed no change in total ERK and no
difference in pERK at any timepoint following IGF (E3R) treatment (Figure 3.12 B). α-tubulin
was used as a loading control as β-actin is a similar molecular weight as ERK.

3.2.6 IGF IR forms heterodimers with EGFR and Insulin R in PC-3M-luc2 cells
The IGF IR has the ability to form heterodimers with other tyrosine kinase receptors. The
interaction of the IGF IR with EGFR or IR following IGF (E3R) treatment was assessed by
immunoprecipitation (IP) followed by Western blotting (Figure 3.10). PC-3M-luc2 cells (1 x
10^6 cells/dish) were treated (+) for 10 min with 100 ng/ml IGF (E3R) or left untreated (-). Cell
lysates were incubated with Sepharose G beads pre-incubated with antibodies to pIGF IR,
IGF IR, pEGFR, EGFR, pIR and IR. Immunoprecipitates were fractionated by 4 - 20 % (w/v)
SDS-PAGE, transferred to nitrocellulose membrane and probed with the above mentioned
antibodies. Cell lysates from untreated (-) and treated (+) PC-3M-luc2 cells were used as
controls for protein expression. Cell lysate incubated with Sepharose G beads only were
used as a negative control (N). IGF IR and pIGF IR pulldown showed that the IGF IR
interacted with the EGFR. IGF (E3R) had no effect on levels of total or phosphorylated
EGFR. When the EGFR and pEGFR were used to pull down the IGF IR and pIGF IR no
increase in IGF IR or pIGFR was seen. The IGF IR was also shown to interact with the IR.
Upon stimulation with IGF (E3R), an increase in pIR was seen whereas total IR levels
remained constant. When the IR and pIR were used to pull down the IGF IR and pIGFR, an
increase in pIGFR was seen (Figure 3.13).

3.2.7 Proliferation of PC-3M-luc2 cells in response to EGF
As PC-3M-luc2 cells express the EGFR, which can form heterodimers with the IGF IR, cell
proliferation in response to EGF ligand was examined using BrdU assay (Figure 3.14). PC-
3M-luc2 cells (3 x 10^5 cells/well) were treated in triplicate with EGF (0 - 100 ng/ml) for 48 h.
Controls were treated with the vehicle concentration equivalent to the highest dose of EGF
(PBS containing 0.1 % (w/v) BSA). Proliferation is expressed as % of control where control
is taken as 100 %. EGF did not have a statistically significant effect on cell proliferation.

3.2.8 EGFR and IGF IR activation following EGF treatment in PC-3M-luc2 cells
As IGF IR forms heterodimers with EGFR, PC-3M-luc2 cells (1.5 x 10^5 cells/well) were
treated with 100 ng/ml EGF for 0 - 60 min to determine if the EGFR cross activated the IGF
IR. PC-3M-luc2 whole cell lysates were fractionated by 4 - 20 % (w/v) SDS-PAGE,
transferred to nitrocellulose membrane and probed with anti-EGFR, anti-pEGFR (Tyr 1173),
anti-IGF IR(β), anti-pIGF IR (Tyr 1131) and anti-β-actin antibodies (Figure 3.15).
Figure 3.12 A & B: Phosphorylation of ERK is not induced by IGF. 1.5 \times 10^5 cells/well of PC-3M-luc2 cells were treated with 100 ng/ml IGF I (A) or IGF (E3R) (B) for 0 - 60 min. Cells were lysed and 25 µg of total cell protein was fractionated by 4 - 20% SDS-PAGE, transferred onto nitrocellulose and probed with an anti-phospho-ERK (Thr202/Tyr 204), anti-ERK or an anti α-tubulin antibody. Position of molecular weight marker is indicated (kDa). Western blots are representative of two independent experiments.
**Figure 3.13: IGF IR forms heterodimers with the EGFR and IR.** PC-3M-luc2 cells were treated with 100 ng/ml IGF (E3R) for 10 min. Heterodimers were immunoprecipitated with the relevant antibody coupled to Sepharose G beads for 2 h at 4°C. Immunoprecipitates (IP) were fractionated by 4 - 20 % (w/v) SDS-PAGE and transferred to nitrocellulose. Heterodimers were identified by Western blotting (WB) with antibodies indicated. Immunoprecipitates (IP) of untreated (-), treated (+) cells and a negative control (N) incubated with Sepharose G beads only are shown. Cell lysates from untreated (-) and treated (+) cells are shown as controls. Position of molecular weight marker is indicated (kDa). IP and Western blots are representative of three independent experiments.

<table>
<thead>
<tr>
<th>kDa</th>
<th>IP</th>
<th>Lysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>260</td>
<td>IGF IRβ</td>
<td>IGF (E3R)</td>
</tr>
<tr>
<td>135</td>
<td>pIGF IR</td>
<td>WB: EGFR (175 kDa)</td>
</tr>
<tr>
<td>260</td>
<td>EGFR</td>
<td>IP: IGF IRβ</td>
</tr>
<tr>
<td>135</td>
<td>p EGFR</td>
<td>WB: p EGFR (175 kDa)</td>
</tr>
<tr>
<td>135</td>
<td>EGFR</td>
<td>IP: pEGFR</td>
</tr>
<tr>
<td>95</td>
<td>IGF IRβ</td>
<td>WB: IR (95 kDa)</td>
</tr>
<tr>
<td>135</td>
<td>p EGFR</td>
<td>IP: pEGFR</td>
</tr>
<tr>
<td>95</td>
<td>IGF IR</td>
<td>WB: p IGF IR (95 kDa)</td>
</tr>
<tr>
<td>135</td>
<td>IR</td>
<td>IP: IGF IRβ</td>
</tr>
<tr>
<td>95</td>
<td>IR</td>
<td>WB: IR (95 kDa)</td>
</tr>
<tr>
<td>135</td>
<td>p IR</td>
<td>IP: pIR</td>
</tr>
<tr>
<td>95</td>
<td>p IGF IR</td>
<td>WB: p IGF IR (95 kDa)</td>
</tr>
</tbody>
</table>
Figure 3.14: PC-3M-luc2 cells do not proliferate in response to EGF. $3 \times 10^3$ PC-3M-luc2 cells were treated in triplicate for 48 h with EGF and proliferation was assayed by BrdU incorporation. Proliferation is expressed as % of control where control is 100 %. Controls were treated with the vehicle concentration equivalent to the highest dose of EGF. Data ($n = 3$) expressed as the mean ± SEM and analysed by one-way ANOVA ($p = \text{ns}$).
EGF stimulated EGFR activation after 20 min and EGFR remained activated up to 60 min post EGF stimulation (Figure 3.15). However, EGF did not activate the IGF IR (Figure 3.15). Total EGFR and IGF IR levels did not change following stimulation with EGF. β-actin shows equal loading in all lanes.
Figure 3.15: EGF activates the EGFR but not the IGF IR. $1.5 \times 10^5$ cells/well of PC-3M-Iuc2 cells were treated with EGF (100 ng/ml) for 0 - 60 min. 25 µg lysate was fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and then probed for EGFR, pEGFR (Tyr 1173), IGF IR, pIGF IR (Tyr 1131) or β-actin as a loading control. Position of molecular weight markers is indicated (kDa). Western blots are representative of three independent experiments.
3.3 Discussion

The IGF IR is a tyrosine kinase receptor involved in cell growth, differentiation and proliferation (Kiepe et al., 2005). In this study, staining of prostate cancer TMAs with an IGF IR antibody showed IGF IR staining in the membrane and cytoplasm of prostate cancer tissue. Membrane and cytoplasmic staining has been noted previously in human prostate tumours (Liao et al., 2005), but the significance of cellular localization in relation to clinical parameters remains unknown. In the current study, membrane IGF IR levels were increased in prostate tumours relative to BPH tissue or normal tissue. Membrane IGF IR levels were highest in Gleason grade 3, but decreased in Gleason grade 5 prostate cancer tissues. This pattern of IGF IR expression was also seen by Liao et al. (2005), where IGF IR expression was high in low Gleason grade tissue and low in high Gleason grade tissue. However, other groups have shown that IGF IR levels remain elevated in primary prostate and metastatic disease (Hellawell et al., 2002). Down-regulation of the IGF IR has been seen in advanced breast cancer relative to control tissue (Schnarr et al., 2000). An exact reason for down-regulation of the IGF IR in advanced cancers remains unclear. One explanation may be the role of IGF IR in differentiation. Early grade tumours are well differentiated whereas more aggressive tumours are poorly differentiated. IGF IR mRNA and protein decreased significantly as prostate epithelial cells underwent transformation to malignancy (Tennant et al., 1996a). Plymate et al. (1997) examined the effect of re-expression of the IGF IR in the highly malignant androgen independent M12 subclone of the P69SV40T immortalised prostate tumour cell line. Expression of the IGF IR in this M12 cell line reduced anchorage independent growth in vitro and tumour growth in vivo, but these cells had increased proliferative response to IGF I in vitro. The authors suggested that the IGF IR may have a dual function in tumours. High IGF IR expression may inhibit tumour growth, but induce differentiation. Whereas, low receptor number may induce proliferation (Plymate et al., 1997). This may explain the high level of membrane IGF IR seen in Gleason grade 3, which is well differentiated, relative to Gleason grade 5 which is poorly differentiated. Low levels of IGF IR seen in Gleason grade 5 may imply a more malignant phenotype. A second explanation for decreased IGF IR expression in higher grade prostate cancer may be due to invasiveness of cancer cells. It has been shown in colorectal neoplasia that decreased IGF IR was found at invasive foci along with loss of cell adhesion (Allison et al., 2007). Knockdown of the IGF IR in IGF IR overexpressing MCF-7 breast cancer cells led to an increase in motility and decreased ability to form aggregates in culture (Pennisi et al., 2002).

IGF IR cytoplasmic staining was also seen in prostate cancer TMAs. No significant difference in cytoplasmic IGF IR was seen between tumour tissue and BPH/normal tissue, whereas cytoplasmic staining in Gleason grade 3 tumours was higher than in BPH/normal tissue or Gleason grade 5. The relevance of cytoplasmic IGF IR in prostate cancer
progression has not been investigated. In TMAs constructed of benign breast biopsies stained for IGF IR there was a positive association between cytoplasmic IGF IR and subsequent risk of breast cancer (odds ratio: 2.47) (Tamimi et al., 2011). However, the association between cytoplasmic IGF IR and prostate cancer risk has not been investigated. The IGF IR can be internalised and recycled to the cell surface following activation by IGF I (Romanelli et al., 2007), leading to regulation of activation of Shc/MAPK signalling (Chow et al., 1998). In Gleason grade 3 prostate cancer tissue, the high level of cytoplasmic IGF IR may be associated with overexpression of membrane IGF IR which may then lead to an increased level of receptor recycling whereas in the higher Gleason grade due to down-regulation of the IGF IR, cytoplasmic IGF IR may not be detected. This is further supported in this study where a positive association between membrane IGF IR and cytoplasmic IGF IR staining was noted (p<0.01). Therefore, an increase in membrane IGF IR staining leads to an increase in cytoplasmic IGF IR staining: Membrane IGF IR shows a stronger relationship with Gleason grade than cytoplasmic IGF IR in prostate cancer (membrane staining p<0.001 vs. p<0.05 cytoplasmic staining), suggesting membrane IGF IR may be more important in prostate tumour development and growth rather than cytoplasmic IGF IR.

In addition to increased IGF IR levels in prostate tumour tissue elevated plasma IGF I levels are associated with increased risk of prostate cancer. In this study, IGF I was measured in the plasma of BPH, Gleason score 5 and Gleason score 7 patients, but no difference in IGF I levels were seen, possibly due to the small number of patient samples (n=33) compared with other studies. Stattin et al (2000) measured plasma IGF I levels in men (n=149) with a diagnosis of prostate cancer between 1 month and 10 years after blood collection. The risk associated with increased IGF I was higher in subjects under the age of 59 years at the time of blood collection, suggesting that elevated IGF I levels may be involved in early pathogenesis of prostate cancer (Stattin et al., 2000). However, it was found that elevated plasma IGF I levels were associated with advanced prostate cancer (Chan et al., 2002). BPH patients may not have been suitable controls for this study as BPH results in enlargement of the prostate due to chronic inflammation, but no association between IGF I levels and BPH has been previously shown (Mantzoros et al., 1997). No correlation was seen between IGF I and PSA or IGF I and Gleason grade which was also reported by Shariat et al (2000) in patients (n=120) who had undergone radical prostatectomy. PAPP-A levels were also measured in the same patient cohort. PAPP-A is a metalloproteinase that cleaves IGFBP 4 (Lawrence et al., 1999) thus modulating IGF availability. In lung cancer, PAPP-A levels are higher in lung cancer patients (n=83) relative to controls (n=33) (Bulut et al., 2009). PAPP-A is expressed in normal prostate epithelium and in seminal fluid as measured by immunoperoxidase and radioimmunoassays (Sjöberg J et al., 1985), but the importance of PAPP-A expression in the male reproductive tract has
not been elucidated. In the current study, plasma PAPP-A measured in BPH, Gleason score 5 and 7 patients showed a significant increase in PAPP-A levels in the Gleason score 5 cohort relative to controls. PAPP-A levels have never been measured in relation to prostate cancer. However, larger numbers would be required to further validate the differences seen in PAPP-A levels. PAPP-A levels were negatively correlated with age, suggesting that measurement of PAPP-A levels in younger men may be a prognostic marker for prostate cancer.

In order to select an *in vivo* model of prostate cancer to evaluate the therapeutic potential of IGF blockade, an appropriate cell line was required. The cell line chosen was the PC-3M-luc2 cells, a human androgen independent cell line derived from a liver metastasis following PC-3 cell injection into the spleen of a mouse (Kozlowski et al., 1984). PC-3M-luc2 cells have been transfected with luciferase to allow non-invasive imaging when growing in mice (IVIS imaging system). Before using the PC-3M-luc2 cells *in vivo* (Chapter 5) they were characterised with respect to expression and activation of IGF pathway components as the protease resistant IGFBP 4 targets the IGF pathway. Therefore, if the PC-3M-luc2 cells were not IGF responsive PC-3M-luc2 tumour growth may not be inhibited by IGF blockade *in vivo*. PC-3M-luc2 cells expressed the IGF IR, which was phosphorylated in response to IGF I treatment. PC-3M-luc2 cells were previously shown to produce IGFBP 3 (Bindukumar et al., 2005) and multiplex PCR analysis confirmed IGFBP 3 expression in this study. IGFBP 4 was detected in PC-3M-luc2 cells at a low level, the majority of which is secreted into the medium. PAPP-A levels were also measured in the PC-3M-luc2 as PAPP-A cleaves IGFBP 4. RT-PCR was used as there is currently no specific antibody for PAPP-A available. The PC-3M-luc2 cells expressed PAPP-A at the mRNA level, but this may not directly reflect protein levels of PAPP-A. PAPP-A protein has been detected in prostate epithelium and in seminal fluid from healthy men (Sjoberg et al., 1985). Serum PAPP-A was measured by ELISA and was shown to be elevated in chronic stable angina. Patients with high levels of PAPP-A had a poorer prognosis compared with those with low levels of the protein (Consuegra-Sanchez et al., 2008). Even though PAPP-A is expressed by men, its role in the male reproductive tract is unknown. Since the PC-3M-luc2 cells are derived from prostate epithelial cells which have been shown to express PAPP-A in men (Sjöberg et al., 1985) this may explain why PAPP-A was detected in these cells. However, this finding has not been previously reported.

Since PC-3M-luc2 expressed the IGF IR, cell proliferation and VEGF_{165} expression was assessed in response to IGF I or IGF (E3R). As PC-3M-luc2 cells express IGFBPs which can bind IGF I and inhibit its actions, IGF (E3R), which is unable to be bound by IGFBPs, was also used. VEGF is an important mediator of angiogenesis, which plays a major role in prostate tumour development and growth by supplying tumour cells with
oxygen and nutrients (Ackerstaff et al., 2004, Kitagawa et al., 2005, Mazzucchelli et al., 2000). There are at least six isoforms of VEGF, VEGF_{121}, VEGF_{145}, VEGF_{165}, VEGF_{183}, VEGF_{189} and VEGF_{206}. VEGF production is stimulated by hypoxia and IGF I (Levy et al., 1996, Menu et al., 2004, Poulaki et al., 2003, Warren et al., 1996). IGF (E3R) stimulation of PC-3M-luc2 cells significantly increased VEGF_{165} (most abundant VEGF isoform) relative to controls, whereas IGF I had no effect possibly due to IGF I being sequestered by IGFBP 3 and IGFBP 4 rendering it inactive and unable to bind the IGF IR. Similarly, IGF (E3R) stimulated PC-3M-luc2 cell proliferation whereas wildtype IGF I had no effect.

In order to determine whether the IGF IR was responsive to IGF I and activated downstream signalling molecules, PC-3M-luc2 cells were stimulated with either IGF I or IGF (E3R). Both IGF I and IGF (E3R) activated the IGF IR. To further investigate the activation of the IGF I pathway, Akt activation was also analysed. High levels of phospho Akt are a strong predictor of biochemical recurrence in prostate cancer (Ayala et al., 2004). By using wortmannin, a PI3K inhibitor which was previously shown to inhibit Akt activation (Powis et al., 1994), it was shown that both IGF I and IGF (E3R) activated Akt specifically via PI3K. Activation occurred rapidly (10 min with IGF I, 5 min with IGF (E3R)) and after 15 minutes the levels of phospho Akt began to decrease. The difference in Akt activation between IGF IGF (E3R) and I may be attributed to IGF I inhibition by IGFBPs. PC-3M-luc2 cells pre-treated with wortmannin showed complete inhibition of Akt phosphorylation following IGF I or IGF (E3R) stimulation. As Akt is involved in stimulating cell proliferation, inhibition of Akt leads to apoptosis (Sekine et al., 2008).

The MAPK pathway is another pathway activated downstream of the IGF IR. Six distinct groups of MAPK have been characterised. The ERK pathway is the most studied, consisting of ERK 1 (p 44) and ERK 2 (p 42) which are linked with cell proliferation and differentiation (Li et al., 2006). Phospho ERK was present in unstimulated PC-3M-luc2 cells and was not increased following IGF I or IGF (E3R) treatment. This suggests that in PC-3M-luc2 cells Akt is the main mediator of IGF signalling via IGF IR. PC-3M-luc2 cells are also PTEN negative (Vlietstra et al., 1998). PTEN inhibits Akt activity, therefore in PTEN negative cells a key regulator of Akt activity is absent which may lead to an Akt dominant pathway. Malik et al (2002) have shown that in high Gleason score tumours, phospho Akt predominates relative to phospho ERK. Phospho ERK was elevated in normal and PIN tissue samples. This suggests that progression of normal prostate tissue to cancerous disease may rely on an increase in Akt and decrease in ERK. This was further supported by protein microarrays, which characterised disease progression at the cancer invasion front in normal, PIN and prostate cancer tissue. Phospho Akt was increased and phospho ERK was suppressed in invasive prostate cancer relative to PIN (Paweletz et al., 2001).
This may explain why no increase in phospho ERK was seen upon IGF I treatment as active ERK decreases in prostate cancer progression.

The IGF IR can form homodimers and heterodimers with other receptors such as the IR (Treadway et al., 1991) and EGFR (Riedemann et al., 2007). IGF (E3R) was used to stimulate the PC-3M-luc2 cells. In PC-3M-luc2 cells, the IGF IR heterodimerised with the IR and EGFR. There was an increase in the interaction between phosphorylated IGF IR and phosphorylated IR following treatment with IGF (E3R) relative to untreated PC-3M-luc2 cells, suggesting that IGF (E3R) also activates the IR in these cells. Johansson et al (2006) have reported IGF IR-IR heterodimers in vascular smooth muscle cells, whereby IGF I increased glucose metabolism and DNA synthesis. Although EGFR-IGF IR heterodimers were identified in PC-3M-luc2 cells, no increase in phosphorylated EGFR was seen upon stimulation with IGF (E3R). Riedemann et al (2007) carried out co-immunoprecipitations in the MDA-MB-468 breast cancer cell line and clinical samples of breast cancer and found that the EGFR interacts with the IGF IR and regulates its stability. Knockdown of the EGFR led to ubiquitinylation (degradation) of the IGF IR in MDA-MB-468 breast cancer cell line. In response to IGF IR blockade, the EGFR pathway may become overactivated in order to overcome the IGF IR inhibition. This effect has been seen upon inhibition of the IGF IR in 5 hepatocellular carcinoma cells (HepG2, Hep3B, HuH7, HuH6 and PLC/PRF5.cells) treated with AVE1642 (mAb), whereby a resistance mechanism is induced via increased phosphorylation of the EGFR and HER3 receptors (Desbois-Mouthon et al., 2009). Therefore, combination therapy to inhibit IGF IR and EGFR may be of value in tumours that develop resistance to IGF IR blockade via EGFR signalling.

The EGFR was shown to be expressed by the PC-3M-luc2 cells and formed heterodimers with the IGF IR. EGFR has also been implicated in prostate cancer growth (Marks et al., 2008). However, PC-3M-luc2 cells did not proliferate in response to EGF. This concurs with a study where PC-3 cells, parental cells of PC-3M did not proliferate in response to EGF treatment. The authors found that phosphorylation of both EGFR and ERBB2 are predictive of prostate cancer cell proliferation in response to EGF (Sheikh et al., 2004). ERBB2 was undetectable in PC-3M cells (Wang et al., 2001). Therefore, proliferation in response to EGF in PC-3M-luc2 cells may be attributed to low levels of ERBB2 as Sheikh et al (2004) suggest that quantifying the sum of EGFR and ERBB2 phosphorylation may identify prostate cancers that grow in response to EGF. EGFR has previously been shown to potentiate the metastatic behaviour of PC-3M cells in vitro (Uysal-Onganer and Djamgoz, 2007). Therefore, EGFR expression in PC-3M-luc2 cells may not be involved in cell proliferation, but in metastatic potential. As the EGFR forms heterodimers with the IGF IR, activation of the IGF IR was assessed following EGF treatment. Contrary to a study where EGF activated the IGF IR in rat hepatocytes (Hallak et al., 2002), EGF treatment did not
activate the IGF IR in PC-3M-luc2 cells. Conversely, IGF I has been shown to activate the EGFR in COS-7 cells (Roudabush et al., 2000). However, in this study, IGF (E3R) did not activate the EGFR.

The key findings from this chapter are that IGF IR expression is important in prostate cancer progression and may be useful in determining early Gleason grade and late Gleason grade prostate cancer tissue as seen in the prostate tumour TMAs as IGF IR levels were higher in Gleason grade 3 prostate tissue and decreased in higher Gleason grade 5. This difference in staining may help in deciding therapeutic strategies for individuals based on receptor status. PC-3M-luc2 cells were selected as suitable for in vivo studies to test protease resistant IGFBP 4 as a strategy to inhibit prostate tumour growth as they express the IGF IR, are IGF (E3R) responsive, express IGFBP 3 and IGFBP 4, the IGFBP 4 protease PAPP-A and Akt signalling is activated downstream of the IGF IR following IGF I treatment. Since the PC-3M-luc2 cells express components of the IGF signalling pathway and proliferate in response to IGF (E3R), utilising a protease resistant IGFBP 4 may inhibit IGF I induced proliferation of these cells in vivo.
Chapter 4
Purification and \textit{in vitro} evaluation of protease resistant IGFBP4 in prostate cancer cells
4.1 Introduction

Due to the success of targeting the HER2 receptor with the monoclonal antibody Herceptin™ (Trastuzumab), focus has turned towards inhibition of the IGF IR. However, there are concerns about targeting the IGF IR due to its importance in normal physiology and its ubiquitous distribution throughout the body. As the IGF IR shares 60% homology with the IR (Ullrich et al., 1986), blocking the IGF IR can inhibit the IR leading to toxicity issues or side effects such as hyperglycaemia which has been reported following treatment with CP-751,871 (IGF IR monoclonal antibody) in patients with refractory solid tumours (Haluska et al., 2007). Numerous pharmaceutical companies have developed molecular targeted reagents to inhibit the IGF IR. The primary drugs being developed involve mAbs and TKIs. In pre-clinical trials, mAbs have shown good results in inhibiting tumour growth in vivo. IMC-A12, a fully humanised monoclonal antibody against the IGF IR developed by ImClone systems, inhibited growth of androgen dependent (LuCaP 35) and androgen independent (LuCaP V5) prostate cancer cells implanted subcutaneously in SCID mice (Wu et al., 2005). IMC-A12 is currently in Phase II trials in patients with mesothelioma (www.clinicaltrials.gov). TKIs or small molecule inhibitors exploit the subtle differences in the kinase domains between the IGF IR and IR, therefore making these drugs more specific for the IGF IR compared with mAbs. NVP-AEW541 was designed by Novartis as an IGF IR specific TKI. In vitro and in vivo NVP-AEW541 down-regulated IGF IR activation in a panel of 10 neuroblastoma cell lines (Tanno et al., 2006), similar to the mAb, IMC-A12 (Rowinsky et al., 2007). NVP-AEW541 in combination with Herceptin™ enhanced the anti-proliferative effect of both drugs compared with either alone in HER2 overexpressing breast cancer cells (MDA-MB-231, BT474, SKBR3 and MCF-7 cells) (Esparis-Ogando et al., 2008). However, NVP-AEW541 did not progress into clinical trials and the reasons for this have not been published.

Targeting the IGF I ligand is an alternative approach to inhibit IGF I induced effects and avoid unwanted effects on insulin signalling. IGFBPs may prove useful as therapeutics for cancer. The IGFBPs are naturally produced within the body, reducing the likelihood of an immune response upon administration. Protease resistant IGFBPs would decrease IGF I bioavailability resulting in inhibition of IGF I activity. IGFBP 4 is the smallest of the binding proteins and contains an N-linked glycosylation site which causes IGFBP 4 to exist in two forms, a 24 kDa non-glycosylated and 28 kDa glycosylated form (Ceda et al., 1991). IGFBP 4 is purely inhibitory to IGF I and does not have any IGF-independent effects compared with other binding proteins. Numerous cancer cell lines have been shown to secrete IGFBP 4. The production of inhibitory IGFBPs by cancer cells or tumours may increase the local reservoir of IGF I thus increasing bioavailability of IGF I in tissues or tumours where PAPP-A is present.
17β estradiol increased IGFBP 4 expression in MCF-7 breast cancer cells (Pratt and Poliak, 1993). Prostate carcinoma cells (androgen independent PC-3 cells) have also been shown to secrete IGFBP 4 (Conover et al., 1995b). As IGFBP 4 inhibits IGF I it suggests an interesting therapeutic approach for blockade of IGF I. Overexpression of wildtype IGFBP 4 in androgen receptor negative M12 prostate cancer cells delayed tumour onset in a subcutaneous prostate cancer model relative to controls (Damon et al., 1998). In a subcutaneous animal model of colon cancer, peritumoural injection of a mammalian vector (pcDNA3) containing wildtype IGFBP 4 cDNA increased apoptosis and decreased the mitotic rate of colon cancer cells (HT-29) relative to controls which received empty vector (Durai et al., 2007). Proteolysis of IGFBP 4 is a regulatory mechanism to control IGF I release and activity. PAPP-A was identified as an IGF dependent IGFBP 4 protease (Lawrence et al., 1999) and cleaves IGFBP 4 into two fragments of 18 kDa and 14 kDa which have decreased affinity for IGF I. PSA can also cleave IGFBP 4 (Rehault et al., 2001), but very little is known about the PSA cleavage site in IGFBP 4. The PAPP-A cleavage site has been well established (Chelius et al., 2000). Zhang et al. (2002a) mutated the PAPP-A cleavage site of rat IGFBP 4 from 119-KHMAKVDRSDMK-133 to 119-AAMAAVADASAMA-133 and demonstrated it was resistant to cleavage by a fibroblast produced protease. This protease resistant IGFBP 4 was used in a porcine model of neointimal hyperplasia where infusion of protease resistant IGFBP 4 into hypercholesterolemic pigs inhibited cell proliferation and neointimal expansion relative to controls (Nichols et al., 2007).

In the Harmey group the equivalent murine protease (PAPP-A) resistant form of IGFBP 4 was constructed using site directed mutagenesis (Appendix 13). The mutated sequence was cloned into a pTriEx4 Neo plasmid which contains a C-ter His-tag coding sequence, N-ter signal sequence to ensure secretion of the cloned protein and a neomycin resistance allowing selection of stable clones (Figure 4.1). Previously, mouse 4T1.2 breast cancer cells were transfected with rat wildtype IGFBP 4, rat protease resistant IGFBP 4 or empty vector and implanted into the mammary fat pad of female BALB/c mice. Overexpression of protease resistant IGFBP 4 in the mice showed a reduction in tumour growth and increased survival relative to controls as well as anti-angiogenic effects (Ryan et al., 2009).

An advantage of using a protease resistant IGFBP 4 to block IGF I is that it is a small molecule compared with other IGFBPs and antibodies and can cross endothelial barriers (Boes et al., 1992). As protease resistant IGFBP 4 cannot be cleaved by PAPP-A it may have a long serum half-life. It has been shown that insertion of 12-mer peptides selected from protective motifs structures (capable of preventing the access of proteases to defined cleavage sites without affecting ligand binding) between a FLAG epitope motif and a
thrombin cleavage site led to peptides resistant to thrombin. The observed resistance to thrombin, resulted in increased resistance to plasma proteases in vitro and to an increase in circulating half-lives in rats (Eldridge et al., 2009). As the protease resistant IGFBP 4 was previously shown to inhibit tumour growth in breast cancer and IGF I has been implicated in prostate cancer, its effects on prostate cancer growth and metastasis were evaluated. The aims of this chapter were to purify protease resistant IGFBP 4 to evaluate it in vitro.

The specific aims of this chapter were:
1. To purify dBP4 protein from HEK 293 cells transfected with dBP4 construct.
2. To determine dBP4 sensitivity to PAPP-A and PSA cleavage.
3. To determine IGF I and IGF II binding affinity of dBP4.
4. To compare the effects of IGF IR TKIs (AG1024 and NVP-AEW541) and dBP4 on proliferation of PC-3M-luc2 cells.
Figure 4.1 Protease resistant mouse IGFBP 4 (dBP4) construct. PAPP-A cleavage site was mutated by site directed mutagenesis to produce a PAPP-A resistant IGFBP 4. The PAPP-A cleavage site is located at residues 119-KHMAKIRDRSKMKV-133. A 6 residue His-tag (HIS) was added at the C-terminus of PAPP-A cleavage site with an enterokinase (EK) site located before the histidine tag (HIS) to allow removal of the His-tag. S represents the signal peptide, which is cleaved upon secretion of dBP4. Amino acid sequence of the full construct is shown in Appendix 13.
4.2 Results

4.2.1 Isolation of protease resistant IGFBP 4 expressing HEK 293 single cell clones

To obtain high levels of protease resistant IGFBP 4 (dBP4) from HEK 293 cells transfected with dBP4, single cell clones were isolated by serial dilution. Single cell clones were incubated in serum free medium for 72 h. Conditioned medium was collected and fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-His-tag antibody (Figure 4.2). The predicted molecular weight of dBP4 is 34 kDa. Clones 5 - 20 expressed dBP4. However, clones 5 - 8 expressed the highest level of dBP4 compared with other clones (Figure 4.2). Clones 5, 6, 7 and 8 were further expanded for dBP4 protein purification.

4.2.2 Purification of dBP4 from HEK 293 single cell clones

Nickel agarose column was used to purify dBP4 protein. dBP4 is a 6 x His-tagged protein allowing for dBP4 to interact with nickel ions covalently linked to the agarose beads. Flow through from each step of the column was retained in order to determine dBP4 recovery. Samples from flow through of conditioned medium, wash buffer, binding buffer, elution buffer and concentrated elution buffer were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-IGFBP 4 or anti-His-tag antibody (Figure 4.3 A & B, respectively). Figure 4.3 A & B show that dBP4 was not present in the conditioned medium flow through (lane 1), wash buffer (lane 2) and binding buffer (lane 3). dBP4 was only present in the elution buffer and concentrated elution buffer (lane 4 & 5, respectively) indicating that the dBP4 loaded bound to nickel agarose and did not elute with the washes. In order to remove any other impurities that may be present Fast Protein Liquid Chromatography (FPLC) was used to further purify dBP4. A Superdex 200 column was used which purifies proteins based on size exclusion. Based on the size of dBP4 (28 - 34 kDa), it was predicted that dBP4 should be eluted after 12 - 13ml (Figure 4.4). dBP4 was dialysed into a running buffer (10 mM HEPES, 150 mM NaCl and 1 mM CaCl2, pH 7.5) to remove imidazole from elution buffer. 500μl of running buffer containing dBP4 was injected into the FPLC and samples were collected and kept on ice for further analysis using Western blot. Figure 4.4 represents the chromatogram obtained from the FPLC, an increase in absorbance at 280 nm is seen when protein has eluted from the column. Peak absorbance at 280 nm was seen after 12 ml volume.

To identify samples containing dBP4, samples from the FPLC were pooled in pairs and fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-His-tag antibody (Figure 4.5). A His-tagged protein Masi (E3 ubiquitin ligase) (provided by Dr. Heidi Daxeckler), was used as a positive control.
**Figure 4.2: dBP4 is expressed by HEK 293 single cell clones.** Single cell clones 1 - 20 were isolated by serial dilution. Concentrated, conditioned medium was collected after 72 h. Conditioned medium was fractionated by 4 - 20% (w/v) SDS-PAGE, transferred to nitrocellulose and then probed for dBP4 expression with anti-His-tag antibody. Position of molecular weight marker is indicated (kDa).
Figure 4.3: Nickel agarose purification of dBP4. dBP4 was isolated from conditioned medium of dBP4-transfected HEK 293 cells by nickel agarose chromatography. Samples were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and probed with anti-His-tag (A) or anti-IGFBP 4 antibody (B). In A & B, lane 1 shows conditioned medium flowthrough, lane 2 shows wash buffer flowthrough, lane 3 shows binding buffer flowthrough, lane 4 shows elution buffer and lane 5 shows concentrated elution buffer (20 x). Position of molecular weight marker is indicated (kDa).
Following nickel agarose column purification, dBPs were further purified using size exclusion FPLC. dBPs were dialysed into a running buffer (10 mM HEPES, 150 mM NaCl and 1 mM CaCl₂, pH 7.5) using Centricon YM-3 filters to a volume of 500 µl and applied to Superdex 200 column and eluted with 10 mM HEPES, 150 mM NaCl and 1 mM CaCl₂, pH 7.5 in 1 ml fractions. The x-axis represents the elution volume and the y-axis represents the UV absorbance (A₂₈₀).
dBP4 was present in fractions 14 - 19 (Figure 4.5). In order to check whether impurities from the nickel agarose column had been removed by FPLC, silver staining was carried out. Fractions from FPLC containing dBP4 were pooled, fractionated by SDS-PAGE and silver stained (Figure 4.6). Purified dBP4 was present in the pooled fractions 14 - 19 (lane 1). A band at approximately 66 kDa (lane 1) was also present in the commercially available IGFBP 4 (lane 2) and is probably indicative of serum albumin contamination.

4.2.3 Cleavage of dBP4 by PAPP-A and PSA

The PAPP-A cleavage site of mouse IGFBP 4 was mutated from 119-KHMAKVRDIRSKMK-133 to 119-AAMAADVADASAMA-133. To test dBP4 resistance to PAPP-A and PSA cleavage, dBP4 was incubated with PAPP-A (a gift from Dr. Claus Oxvig) or PSA overnight at 37°C. Recombinant human IGFBP 4 was used as a positive control for protein cleavage. Intact IGFBP 4/dBP4 and cleavage fragments were identified with anti-IGFBP 4 antibody (Figure 4.7). PAPP-A cleavage of IGFBP4 generates fragments of 18 kDa and 14 kDa. In the presence of PAPP-A, dBP4 was not cleaved. Recombinant IGFBP 4 was cleaved by PAPP-A (18 kDa and 14 kDa) (Figure 4.7). In the presence of PSA, dBP4 was cleaved into two fragments of approximately 17 and 10 kDa in size whereas recombinant IGFBP 4 was cleaved into one fragment (~26 kDa). Untreated dBP4 and recombinant IGFBP 4 remained intact. Since PSA cleavage of dBP4 generated different sized fragments compared with recombinant IGFBP 4, this may suggest that mutation of the PAPP-A site has increased PSA cleavage due to either a change in amino acid sequence or a change in conformation.

4.2.4 IGF binding to dBP4

An IGF-binding ELISA method was developed to test the binding capacity of dBP4 for IGF I and IGF II. A 96 well plate was coated with 50 ng/ml IGF I, IGF II or VEGF165. To demonstrate specificity of dBP4, binding to VEGF165 was assayed as a non-specific ligand as dBP4 does not bind VEGF165. As an additional control, dBP4 was pre-incubated with IGF I/II for 30 min. Pre-incubation with IGF I/II prevented dBP4 binding to IGF I/II coated wells further demonstrating specificity of IGF/dBP4 interaction. dBP4 (500 ng/ml) binding was detected by incubation with HRP substrate and absorbance read at 450 nm (Figure 4.8). An increase in absorbance was not seen following addition of dBP4 to IGF I or IGF II coated wells. Therefore, dBP4 did not bind IGF I or IGF II using this assay. Statistical analysis could not be carried out on data as it is only representative of n = 2.
Figure 4.5: dBP4 in FPLC fractions. Following size exclusion chromatography, paired fractions were pooled, concentrated and fractionated by 4 - 20% (w/v) SDS-PAGE, transferred to nitrocellulose and dBP4 was detected using anti-His-tag antibody. Masi (RN181) is a His-tagged E3 ubiquitin ligase used as a positive control. Position of molecular weight marker is indicated (kDa).
Figure 4.6: Silver stain of purified dB4. FPLC purified dB4 was fractionated by 4 - 20 % (w/v) SDS-PAGE and silver staining carried out to assess protein purity. Lane 1 shows purified dB4 and lane 2 shows commercially available recombinant IGFBP 4 (20 μg, 28 kDa). Position of molecular weight marker is indicated (kDa).
Figure 4.7: dBP4 sensitivity to PAPP-A and PSA. dBP4 or recombinant IGFBP 4 (20 µg) were incubated overnight at 37°C with either PAPP-A (2 µi) or PSA (20 ng). Samples were fractionated on 4 - 20 % (w/v) SDS-PAGE and transferred to nitrocellulose. dBP4 and recombinant IGFBP 4 cleavage was analysed by anti-IGFBP 4 antibody. Recombinant IGFBP 4 was used as a positive control for protein cleavage. Position of molecular weight marker is indicated (kDa). Western blots are representative of three independent experiments.
Figure 4.8: dBP4 binds IGF I. An ELISA based method was used to determine dBP4 binding affinity for IGF I and IGF II. A 96 well plate was coated with 50 ng/ml IGF I, IGF II or VEGF$_{165}$ as a non-specific ligand. dBP4 was pre-incubated with either IGF I (50 ng/ml) or IGF II (50 ng/ml) for 30 min prior to addition to the plate as a second control for dBP4 binding. VEGF$_{165}$ was used as a non-specific control. dBP4 binding to IGF-coated wells was detected by incubation with HRP-labelled anti His-tag antibody and bound antibody was detected using HRP substrate and absorbance read at 450 nm ($A_{450}$). Uncoated wells were incubated with dBP4 or buffer alone. Data (n =2) expressed as the mean. Statistical analysis was not carried out.
4.2.5 Effect of tyrosine kinase inhibitors on PC-3M-luc2 proliferation

To determine whether blockade of the IGF pathway inhibits cell proliferation tyrosine kinase inhibitors AG1024 (Figure 4.9) (Merck, Nottingham, UK) and NVP-AEW541 (Figure 4.10) (provided by Dr. Andrew Kung, DFCI, MA, USA) were used to test if IGF IR inhibition blocks PC-3M-luc2 proliferation. AG1024 inhibits both the IR and IGF IR (Párrizas et al., 1997a) whereas NVP-AEW541 is an IGF IR specific inhibitor (Garcia-Echeverria et al., 2004). PC-3M-luc2 (5 x 10^3 cells/well) were treated with AG1024 or NVP-AEW541 (0 - 5 μM) in the absence or presence of IGF 1 (100 ng/ml) or IGF (E3R) (100 ng/ml) for 48 h. Cell proliferation and cytotoxicity was assayed by MTS assay. Controls were treated with the vehicle concentration equivalent to the highest dose of IGF and/or drug (DMSO (5 μM) and PBS / 0.1 % (w/v) BSA (IGF 1) or PBS / 10 mM HCl / 0.5% (w/v) BSA (IGF (E3R)) and DMSO (5 μM) or DMSO (5 μM) alone), IGF 1 or IGF (E3R) alone (100 ng/ml). Proliferation is expressed as % of control where control is taken as 100 %. PC-3M-luc2 cells treated with AG1024 alone showed a significant decrease in proliferation at 0.5 μM (p<0.002), 1 μM (p<0.001) and 5 μM (p<0.0001) relative to controls. Treatment of PC-3M-luc2 cells with IGF 1 or IGF (E3R) caused a significant increase in proliferation relative to controls (p<0.017 and p<0.05, respectively). AG1024 significantly inhibited IGF-induced proliferation at 0.1 μM (p<0.007), 0.5 μM (p<0.002), 1 μM (p<0.002) and 5 μM (p<0.0001) relative to PC-3M-luc2 cells treated with IGF 1 alone. AG1024 significantly inhibited IGF (E3R) induced proliferation at doses of 0.1 μM (p<0.006), 0.5 μM (p<0.0001), 1 μM (p<0.0001) and 5 μM (p<0.0001) relative to PC-3M-luc2 cells treated with IGF (E3R) alone. AG1024 inhibited IGF-induced proliferation at a dose of 0.1 μM.

Figure 4.10 shows treatment of PC-3M-luc2 cells with IGF 1 or IGF (E3R) and NVP-AEW541. PC-3M-luc2 cells treated with NVP-AEW541 alone showed a significant reduction in proliferation at a dose of 5 μM relative to controls (p<0.001). IGF 1 or IGF (E3R) significantly increased proliferation of PC-3M-luc2 cells relative to controls (p<0.045 and p<0.031, respectively). NVP-AEW541 significantly inhibited IGF-induced proliferation at 1 μM (p<0.021) and 5 μM (p<0.001) relative to PC-3M-luc2 cells treated with IGF 1 alone. Similarly, NVP-AEW541 significantly inhibited IGF (E3R) induced proliferation at doses of 1 μM (p<0.017) and 5 μM (p<0.001) relative to PC-3M-luc2 cells treated with IGF (E3R) alone. Therefore, NVP-AEW541 inhibited IGF-induced proliferation at a dose of 1 μM. Previously, it was shown by BrdU (Figure 3.8) that PC-3M-luc2 cells do not proliferate in response to IGF 1, however the MTS assay showed an increase in proliferation following IGF 1 treatment (Figure 4.9 and Figure 4.10). This difference may be attributed to seeding density as the BrdU required 3 x 10^3 cells vs. 5 x 10^3 cells in the MTS assay.
Figure 4.9: AG1024 inhibits IGF-induced proliferation in PC-3M-luc2 cells. $5 \times 10^3$ cells/well PC-3M-luc2 cells were treated with tyrosine kinase inhibitor AG1024 (0.1 - 5 µM) with or without 100 ng/ml IGF I and IGF (E3R) in triplicate for 48 h and proliferation assayed by MTS assay. Proliferation is expressed as % of control where control is taken as 100%. Controls were treated with the vehicle concentration equivalent to the highest dose of IGF and/or drug. Data (n = 3) expressed as the mean ± SEM and analysed by one-way ANOVA followed by LSD post hoc correction. *p<0.05 vs Control, **p<0.01 vs Control, $$$p<0.001$ vs. IGF I, $$$$p<0.001$ vs. IGF (E3R).
In addition, the MTS assay is based on metabolic activity, therefore IGF I may affect metabolic activity as well as DNA synthesis (BrdU assay).

4.2.6 Effect of dBP4 on PC-3M-luc2 cell proliferation
dBP4 was used to block IGF I induced proliferation in PC-3M-luc2 cells. PC-3M-luc2 (5 × 10^3 cells/well) were treated with dBP4 (2.5 μg/ml) in the absence or presence of IGF I (50 ng/ml) for 48 h and cell proliferation and cytotoxicity was assayed by MTS assay. Controls were treated with vehicle (PBS). Proliferation is expressed as % of control where control is taken as 100 %. Treatment of PC-3M-luc2 cells with dBP4 or dBP4 plus IGF I had no significant effect on inhibiting proliferation (Figure 4.11). However, it was technically impossible to show an effect of dBP4 on proliferation in vitro as IGF I had no effect on PC-3M-luc2 cell proliferation in the first instance. Previously, IGF (E3R) increased proliferation, but it cannot be bound by IGFBPs therefore it could not be used for this experiment. A lower dose of IGF I was used in this experiment compared with Figure 4.9 and Figure 4.10, this may have contributed to IGF I having no effect on proliferation.

4.2.7 Effect of dBP4 on angiogenesis in human endothelial cells
An Angiokit was purchased from TCS Cell Works (Buckingham, UK) which contained a 24-well plate of early stage tubule forming human endothelial cells. The effect of dBP4 on angiogenesis (tubule formation) was assessed by staining the cells for CD31+ (work carried out by Dr. Constanze Schadow). Human endothelial cells were treated in triplicate for 10 days with VEGF (10 ng/ml, positive control), an angiogenesis inhibitor, Suramin (20 μM, negative control), IGF I (100 ng/ml) or IGF I (100 ng/ml) pre-incubated with dBP4 (2.5 μg/ml) for 30 min. Control wells were untreated. Medium was changed every 3 days along with fresh treatments. Figure 4.12 A shows untreated endothelial cells with multiple tubules formed. VEGF-treated endothelial cells showed a greater number of tubules (Figure 4.12 B). Suramin-treated endothelial cells showed a decrease in tubule formation relative to untreated controls and VEGF-treated cells (Figure 4.12 C). IGF I treated endothelial cells showed tubule formation, but tubule formation was less than that seen in VEGF-treated endothelial cells (Figure 4.12 D) and IGF I pre-incubated with dBP4 showed a large decrease in tubule formation relative to all other treatments (Figure 4.12 E).

Images from 3 different fields of view were taken from each triplicate and the Angiosys software was used to determine the number of tubules formed following each treatment. Dr. Silvia Napoletano carried out statistics, which were performed between each biological triplicate for each treatment group.
Figure 4.10: NVP-AEW541 inhibits IGF-induced proliferation in PC-3M-luc2. \(5 \times 10^3\) cells/well PC-3M-luc2 cells were treated with tyrosine kinase inhibitor, NVP-AEW541 (0.1 - 5 \(\mu\)M) with or without 100 ng/ml IGF I and IGF (E3R) in triplicate for 48 h and proliferation assayed by MTS assay. Proliferation is expressed as % of control where control is taken as 100 %. Controls were treated with the vehicle concentration equivalent to the highest dose of IGF and/or drug. Data (n = 3) expressed as the mean ± SEM and analysed by one-way ANOVA followed by LSD post hoc correction. *p<0.05 vs. Control, ***p<0.001 vs. Control, $p<0.05$ vs. IGF I, $$$p<0.001$ vs. IGF I, †p<0.05 vs. IGF (E3R) and ‡‡‡p<0.001 vs. IGF (E3R).
Figure 4.11: Effect of dBP4 on PC-3M-luc2 proliferation. $5 \times 10^3$ cells/well PC-3M-luc2 cells were treated with dBP4 (2.5 µg/ml) with or without 50 ng/ml IGF I in triplicate for 48 h and proliferation assayed by MTS assay. Proliferation is expressed as % of control where control is taken as 100%. Data ($n = 3$) are expressed as the mean ± SEM and analysed by one-way ANOVA ($p = ns$).
Figure 4.12: dBp4 inhibits tubule formation by human endothelial cells. Early stage tubule forming human endothelial cells were treated in triplicate for 10 days with VEGF (positive control, 10 ng/ml), angiogenesis inhibitor, Suramin (negative control, 20 μM), IGF I (100 ng/ml) or IGF I (100 ng/ml) pre-incubated with dBp4 (2.5 μg/ml) for 30 min. Controls were untreated. Tubule formation was assessed following staining for CD31. Representative images from each treatment are shown. (A) shows untreated endothelial cells, (B) shows VEGF (positive control, 10 ng/ml) treated endothelial cells, (C) shows Suramin (negative control, 20 μM) treated endothelial cells, (D) shows IGF I (100 ng/ml) treated endothelial cells and (E) shows IGF I (100 ng/ml) and dBp4 (2.5 μg/ml) treated endothelial cells. (Original magnification 200 ×)
A significant increase in tubule number was noted following treatment with VEGF (10 ng/ml, positive control) relative to untreated controls (278 ± 16 vs. 165.8 ± 13.9, p<0.001). No significant difference in tubule number was seen between controls or IGF I treated wells. It is likely that the growth factor cocktail provided with the Angiokit already contained IGF I so the addition of further IGF I may have had no effect. However, TCS Cellworks could not confirm the absence or presence of IGF I in the cocktail. Suramin (20 μM, negative control) treatment significantly reduced tubule number relative to untreated controls (81.6 ± 5.2 vs. 165.8 ± 13.9, p<0.01). IGF I (100 ng/ml) and dBP4 (2.5 μg/ml) treatment significantly reduced tubule number relative to untreated controls (11.4 ± 2.2 vs. 165.8 ± 13.9, p<0.001). IGF I (100 ng/ml) and dBP4 (2.5 μg/ml) treatment significantly decreased tubule number relative to IGF I (100 ng/ml) treated endothelial cells (11.4 ± 2.2 vs. 140.3 ± 17.8, p<0.001) (Figure 4.13). These results further support IGF I presence in the growth factor cocktail as dBP4 significantly reduced tubule formation relative to controls.
Figure 4.13: dBp4 decreases tubule number in endothelial cells relative to untreated controls or VEGF-treated endothelial cells. A 24-well plate of early stage tubule forming human endothelial cells were treated in triplicate for 5 days with VEGF (positive control, 10 ng/ml), an angiogenesis inhibitor, Suramin (negative control, 20 μM), IGF I (100 ng/ml) or IGF I (100 ng/ml) pre-incubated with dBp4 (2.5 μg/ml) for 30 min. Controls were untreated. Tubule formation was assessed following staining for CD31+. 3 fields of view were taken for each triplicate well and AngioSys Software was used to calculate the number of tubules. Data are expressed (n=3) as mean ± SEM. A one-way ANOVA was carried out on triplicates followed by Tukey post hoc correction. **p<0.01 vs. Control, ***p<0.001 vs. Control, $$$p<0.001 vs. IGF I.
4.3 Discussion

The IGF pathway plays a central role in transformation and tumourigenesis (Kaleko et al., 1990). Due to the involvement of the IGF pathway in cancer it has proved an attractive therapeutic target. Numerous therapeutics have been developed against the IGF IR, but the IGF IR forms hybrid receptors with the IR (Johansson and Arqvist, 2006) and shares 60 % homology with this receptor (Ullrich et al., 1986). Inhibition of the IGF IR can also inhibit the IR leading to complications such as the clinical syndrome of insulin resistance which can lead to diabetes (Araújo et al., 2005). New therapies are required to overcome the issue of IR blockade. In this study, the ligand IGF I was chosen as the target. IGFBPs are naturally occurring regulators of IGF I/II activity. Exploiting the ability of the IGFBPs to inhibit IGF I led to the development of a PAPP-A resistant form of IGFBP 4 (dBP4).

HEK 293 cells were chosen for dBP4 production as they are a mammalian cell line which do not produce endogenous IGFBP 4 and would allow for correct folding, glycosylation and secretion of dBP4 facilitating protein purification. The addition of a C-ter His-tag allowed differentiation of dBP4 vs. endogenous IGFBP 4 and facilitated purification by nickel-agarose affinity chromatography. The nickel column was an efficient method of purifying dBP4 from the conditioned medium, as none of the protein was lost during each step. FPLC was used to further purify dBP4. The FPLC purification can be monitored allowing for the detection of proteins eluting from the column. Samples correlating to an increase in absorbance were further analysed using Western blot to confirm the presence of dBP4. The silver stained gel allowed us to assess the purity of the samples. The band that appeared at 66 kDa was that of albumin which was also seen in the commercially available IGFBP 4. The presence of albumin in the dBP4 sample is indicative of residual serum in medium and the commercially available IGFBP 4 is reconstituted in PBS containing 0.1 % BSA. This method of purification has been used previously to purify His-tagged proteins (Overgaard et al., 2001).

PAPP-A is the main IGFBP 4 protease (Lawrence et al., 1999). To assess that dBP4 was resistant to PAPP-A a cleavage assay was carried out. PSA which is elevated in prostate cancer patients was also tested as it is known to cleave IGFBP 4 (Rehault et al., 2001). dBP4 was resistant to PAPP-A cleavage but was cleaved by PSA. Therefore, PSA cleaves dBP4 at a different site to PAPP-A with the major fragment occurring at 10 kDa. The PSA cleavage site in IGFBP 4 has not been established. The PSA cleavage site in IGFBP 3 is known to occur at Tyr159 (Okabe et al., 1999) in the mid region of the protein which differs between each binding protein. The substrate specificity of PSA has been investigated to determine preferred sequences for PSA cleavage. Tyrosine was reported as a preferred site of hydrolysis. The consensus sequence SS(Y/F)YSjSG was determined as the sequence for most efficient peptide hydrolysis by PSA (Coombs et al., 1998), but this consensus...
sequence is not found in dBP4 (see Appendix 13). However, PSA cleavage of dBP4 led to an increased number of fragments relative to recombinant IGFBP 4. Therefore, mutation of the PAPP-A sequence may have introduced more cleavage sites for PSA due to either the change in amino acid sequence or a change in conformation. Further mutation of dBP4 to generate a PAPP-A and PSA resistant variant could be pursued as a therapeutic in prostate cancer if the mutation did not affect IGF binding capacity. However, the cells used in this study, PC-3M-luc2 cells, do not produce PSA.

dBP4 binding of IGF I and IGF II was assessed to determine if mutation of the PAPP-A cleavage site altered binding to IGF I and IGF II. dBP4 was not shown to bind IGF or IGF II in ELISA based assay. However, Dr. Anthony Chubb previously carried out Biacore analysis of dBP4 to determine the binding affinity of dBP4 to IGF I relative to wildtype IGFBP 4. The binding affinity for IGF I by wildtype IGFBP 4 or dBP4 was comparable (3.9 x 10^{-9} M vs. 3.5 x 10^{-9} M, respectively) (Chubb and Harmey, unpublished data). IGFBP 4 binds IGF I and IGF II with equal affinity (Kiefer et al., 1992). The IGF binding domain for IGFBP 4 consists of the N-terminal Leu^{72} - Ser^{91} and the C-terminal Cys^{205} - Val^{214}. They are important for correct folding and high affinity binding of IGFs (Qin et al., 1998). The PAPP-A cleavage site is located at Lys^{119} - Lys^{133} therefore mutation of the PAPP-A site should not alter the IGF binding domain as it is outside the IGF binding domains. Molecular modelling predicted that dBP4 should therefore bind both IGF I and IGF II.

dBP4 was compared with the commercially available IGF IR tyrosine kinase inhibitors AG1024 and NVP-AEW541. AG1024 is a tyrphostin, a synthetic protein tyrosine kinase inhibiting receptor autophosphorylation. AG1024 dose dependently inhibited cell proliferation of LNCaP cells, an androgen dependent prostate cancer cell line (Thomas et al., 2009). NVP-AEW541 is an IGF IR tyrosine kinase inhibitor. IGF I or IGF (E3R) increased proliferation of PC-3M-luc2 cells relative to controls. Following treatment of PC-3M-luc2 with AG1024 alone a significant decrease in proliferation was reported at doses 0.5 - 5 μM relative to controls. However, when AG1024 and IGF I was compared with IGF I alone, AG1024 and IGF I significantly inhibited IGF I induced proliferation at 0.1 - 5 μM. Therefore, AG1024 inhibited IGF I induced proliferation at a dose of 0.1 μM. In PC-3M-luc2 cells treated with IGF (E3R) and AG1024, a significant decrease in proliferation was reported at doses of 0.1 - 5 μM compared with doses of 0.5 μM - 5 μM AG1024 vs. controls. Therefore, AG1024 inhibited IGF (E3R) induced proliferation at a dose of 0.1 μM. In NVP-AEW541 treatments, IGF I or IGF (E3R) significantly increased proliferation of PC-3M-luc2 cells. NVP-AEW541 alone inhibited proliferation at 5 μM relative to controls. However, when IGF I and NVP-AEW541 was compared with IGF I alone a significant decrease in proliferation was reported at 1 μM and 5 μM. A similar effect was seen when IGF (E3R) and NVP-AEW541 was compared with IGF (E3R) alone. NVP-AEW541 inhibited IGF-induced proliferation at a
dose of 1 μM. Previously, it was shown by BrdU that IGF I did not induce proliferation of PC-3M-luc2 cells (Chapter 3) whereas in the MTS assay a significant increase in proliferation was seen following IGF I treatment. The difference in the effect of IGF I may have been attributed to a higher seeding density used in the MTS assay (5 x 10^3 cells) compared with the BrdU assay (3 x 10^3 cells). Although the MTS assay is useful for quantification of viable cells this assay has a number of limitations. Cells with low metabolic activity must be used in high numbers to detect changes in viability in response to treatments. The assay assumes that metabolic activity is proportional to cell viability, which may not be the case, as treatments may increase, or decrease metabolic activity without affecting cell number. However, this assay is widely used to estimate proliferation (Marrero et al., 1997, Ono et al., 2004, Helguero et al., 2005) and is an accepted technique to assess cytotoxicity, drug sensitivity and cell activation. The MTS assay has advantages over other assays i.e. BrdU incorporation, as it requires less cell manipulation and therefore may reduce errors. The MTS is also based on a metabolic reaction compared with the BrdU assay which directly measures DNA synthesis, therefore IGF I may increase metabolic activity of the PC-3M-luc2 and/or DNA synthesis.

dBP4 binds the IGF I ligand rather than blocking the IGF IR receptor. PC-3M-luc2 cells were treated with IGF I instead of IGF (E3R) as IGF (E3R) cannot be bound to IGFBPs, therefore dBP4 would not have inhibited it. dBP4 should inhibit IGF-induced proliferation. A lower dose of IGF I (50 ng/ml) was used compared with AG1024 or NVP-AEW541 experiments (100 ng/ml) as a 5:1 molar excess of dBP4 was used (50 ng/ml IGF:2.5 μg/ml dBP4) and due to a limited amount of protein the higher dose of IGF I was not used.

IGF (E3R) significantly increased VEGF production in PC-3M-luc2 cells suggesting that dBP4 could have anti-angiogenic effects. In this study, the anti-angiogenic effect of dBP4 is supported by a significant reduction in tubule formation following treatment of human endothelial cells with dBP4 in the presence of IGF I relative to untreated controls or IGF I treated endothelial cells (p<0.001). dBP4 increased apoptosis of endothelial cells within mammary 4T1.2 breast cancer tumours (Ryan et al., 2009).

Assessment of colony formation and invasion of PC-3M-luc2 cells may have provided additional information on the anti-metastatic effect of dBP4 in vitro. Wildtype IGFBP 4 inhibits colony formation and invasion through Matrigel in colon cancer cells sensitive (LS1034 cells) and insensitive to IGF I (Isreo-1 cells) (Diehl et al., 2004). Given the effect of dBP4 on tubule formation and the potential role for it as an anti-angiogenic molecule, future in vitro work should also include the assessment of these parameters.

The key findings from this chapter were dBP4 was effectively purified from HEK 293 single cell clones using nickel column purification followed by FPLC. However, ~ 30 μg/ml of dBP4 was purified, which was insufficient for use in vivo, but was shown to work in vitro.
dBp4 remained insensitive to PAPP-A cleavage, but was cleaved by PSA at different sites to the recombinant IGFBP 4. dBp4 maintained its binding capacity to IGF I. Inhibition of the IGF pathway caused a decrease in PC-3M-luc2 cell proliferation, as seen with two TKIs AG1024 and NVP-AEW541. dBp4 significantly inhibited tubule formation in human endothelial cells suggesting an anti-angiogenic effect. The effect of dBp4 on PC-3M-luc2 cells could not be fully assessed in vitro suggesting that other parameters such as angiogenesis, apoptosis and colony formation may give a better insight into dBp4 effects in vitro.
Chapter 5

In vivo effect of inducible vector expressed protease resistant IGFBP 4 on prostate cancer growth
5.1 Introduction

Pre-clinical models of cancer have proven to be essential in further understanding human cancer and in the development of novel therapeutics. Animal models should recapitulate many aspects of human cancer such as hormone dependency, angiogenesis and tumour-stromal interactions. Advances within the field of molecular and cellular imaging have now allowed researchers to non-invasively measure tumours at serial timepoints from the same individual subject leading to smaller cohorts of experimental animals (Dickson et al., 2007).

One of the most important aspects of testing new therapies pre-clinically is selecting an appropriate model. Transgenic mice and xenografts are commonly used as prostate cancer models. Transgenic mice have been engineered to develop prostate cancer with prostate specific transforming agents in combination with prostate specific promoters. The most commonly used transforming agent is the SV40 T antigens. SV40 oncoproteins inactivate the tumour suppressor p53 thus allowing transformation of the mouse prostate to occur (Mietz et al., 1992). Xenografts allow implantation of human tissue or cells into immunodeficient mice. Nude mice in which a genetic mutation leads to absence of a thymus thus leading to a repressed immune system lacking T cells have long been used for xenograft models of cancer (Rygaard and Poulsen, 1969). NOD SCID Gamma mice are more immunodeficient than nude mice. Subcutaneous injection of human cells has commonly been used to test drugs pre-clinically due to ease of use and large amount of data obtained. However, one drawback to subcutaneous xenograft models is they do not accurately represent clinical cancer as the tumours are growing in a different tissue with a different microenvironment (Stephenson et al., 1992). Orthotopic implantation of human cells is becoming more common where human cancer cells are implanted into the appropriate anatomical site. Orthotopic xenograft models allow investigation of human cancer to determine the influence of microenvironment on growth, behaviour of human tumour cells and metastasis formation (Wang et al., 2005). However, the cell-cell interactions involved within the microenvironment are between the murine host cells and the human tumours cells, which may affect the behaviour of human tumours. The orthotopic prostate cancer model is a better representation of human prostate cancer than subcutaneous models (Stephenson et al., 1992). Interaction of tumour cells with the microenvironment is crucial in tumour development (Liotta and Kohn, 2001).

Bone metastasis is a common feature of advanced stage prostate cancer (Dai et al., 2008). Orthotopic prostate models have been shown to metastasise previously (Yang et al., 1999), but not all animal prostate cancer models develop metastases. In order to establish bone metastases, experimental metastasis models have been established, the most common of which is intracardiac injection of tumour cells via the left ventricle which gives rise to bone metastases (Jenkins et al., 2003, Sasaki et al., 1995).
A second method involves IV injection of tumour cells via the lateral tail vein, which gives rise to either lung or less frequently bone and liver metastases (Lu et al., 2010, Nemeth et al., 1999).

In this study, a xenograft model was chosen. Human prostate cancer cells PC-3M-luc2 dBP4, were implanted into immunodeficient NOD SCID Gamma (NSG) mice to examine the efficacy of dBP4 against prostate cancer. NOD mice were established from inbreeding of cataract prone mice (cataract shionogi mice), which spontaneously exhibited polyuria and glucosuria accompanied by rapid weight loss. Mating pairs were selected and offspring showing spontaneous diabetes and reproductive abilities were further mated. Following 6 generations a stable diabetic strain was established (Makino et al., 1980).

NSG mice are double homozygous for SCID mutation and Interleukin-2 R gamma (IL-2R γ) allelic mutation and were established following 8 back backcross matings between C57BL/6J ynull mice with NOD/Shi-scid mice resulting in NSG mice with no active T-cells, B-cells, natural killer cells, reduced complement activity and macrophage and dendritic cell dysfunction. NSG mice are therefore a more appropriate mouse strain for xenograft implantation compared with nude mice as the reduced immunogenicity improves transplantability of human tissue or cells. NSG mice have been shown to be an excellent recipient for human cell engraftment (Ito et al., 2002).

A Tet-On inducible system was chosen to block IGF I effects in vivo using dBP4. The Tet-On inducible system allows the dBP4 protein to be induced within tumours once established whereas previously dBP4 was constitutively expressed in a 4T1.2 breast cancer model (Ryan et al., 2009). The principle of the Tet-On system is that the gene of interest is only expressed in response to doxycycline. The first component of the Tet-On system is a regulatory protein, which is based on the Tet repressor protein (TetR) produced by E. coli, which negatively regulates expression of genes of the tetracycline-resistance operon. The regulatory protein for the Tet-Off system is based on the TetR protein which is fused to the C-terminal of Herpes simplex virus VP16 domain that converts TetR from a transcriptional repressor to a transcriptional activator (Triezenberg et al., 1988). The resulting hybrid protein is known as tetracycline-controlled transactivator (tTA). The tTA protein is produced by the pTet-Off regulator plasmid. The Tet-On system is similar to the Tet-Off system, but the regulatory protein is a reverse Tet repressor (rTetR) which contains a 4 amino acid change (Gossen et al., 1995). rTTA is encoded by the pTet-On regulator plasmid. Both Tet-Off and Tet-On plasmids contain a neomycin-resistance gene. PC-3M-luc2 cells were initially transduced with the pTet-On plasmid and a stable cell line was established under selection with genetin (G418). The second component of the Tet-On System is the response plasmid, which expresses the gene of interest, in this case dBP4, cloned under control of the Tet response element (TRE). The plasmid that dBP4 was cloned into is pLVX Tight Puro
which contains a puromycin resistance gene, allowing for selection of stable cell line expressing dBP4. Ultimately, a cell line was established containing the regulatory and response plasmids where addition of doxycycline turned on dBP4 expression. The advantages of using the Tet-On inducible system for this study is that it allows tumours to grow and become established before induction of dBP4, which mimics the clinical situation. Other advantages of the Tet-On system are high expression levels (Yin et al., 1996) and no pleiotropic effects as rtTA binds specifically to its target sequences (Harkin et al., 1999). The effect of dBP4 expression on tumour growth in vivo was assessed using subcutaneous, orthotopic and experimental metastasis prostate cancer models.

Tumour growth and metastasis was monitored by bioluminescent in vivo imaging (BLI) as PC-3M-luc2 dBP4 cells express luciferase, which converts luciferin to oxyluciferin and light, which is detected by the IVIS imaging system. Individual mice can be imaged over a defined time course leading to a reduction in animal numbers. Tight correlations between tumour burden and photon emission have been demonstrated (Jenkins et al., 2003). As ATP and oxygen are required along with luciferin for light emission, luciferase provides a quantitative measure of viable cells (Jenkins et al., 2003, Rehemtulla et al., 2000). BLI is quick and relatively inexpensive. Image acquisition time can vary from 1 s - 60 s making it possible to image numerous treatment cohorts in a single session.

The increased availability of small animal imaging technology has facilitated rapid progress in understanding tumour biology and evaluation of novel therapeutics (Chinnaiyan et al., 2000). The overall aim of this chapter is to subclone the dBP4 construct into the Tet inducible system and transduce PC-3M-luc2 with the Tet inducible system (PC-3M-luc2 dBP4). The effect of dBP4 expression on PC-3M-luc2 dBP4 prostate tumour growth and metastasis was assessed in NSG mice.

In Chapter 4, dBP4 inhibited tubule formation in vitro therefore; anti-angiogenic effects are anticipated in vivo. IGF (E3R) increased PC-3M-luc2 cell proliferation in vitro. Therefore, in vivo dBP4 may target PC-3M-luc2 tumour growth in two ways - by blocking tumour cell growth and proliferation directly and indirectly by inhibiting angiogenesis within the tumours.

The specific aims of this chapter were:
1. To clone dBP4 construct into Tet inducible system (PC-3M-luc2 dBP4 cells).
2. To transduce PC-3M-luc2 cells with Tet inducible system and to test the effects of dBP4 expression following addition of doxycycline when tumours were palpable.
3. To test the effect of dBP4 expression on PC-3M-luc2 dBP4 tumour growth in subcutaneous, orthotopic and experimental metastasis models of prostate cancer.
4. To compare the effects of dBp4 expression (mice fed on doxycycline chow) with controls (fed on normal chow) and mice treated with a known tyrosine kinase inhibitor against the IGF IR, NVP-AEW541.

5. To determine the effect of dBp4 expression on serum and tumour IGF I levels, blood glucose and the IGF signalling pathway in vivo compared with controls or NVP-AEW541 treated mice.
5.2 Results

5.2.1 Cloning of dBp4 into pLVX Tight Puro lentiviral vector

In order to subclone dBp4 from the pTriEx4 Neo plasmid into the doxycycline inducible pLVX Tight Puro (lentiviral) vector, restriction enzyme sites for *BamH* (5' end) and *EcoRI* (3' end) were added onto dBp4 construct using dBp4-specific PCR primers. The pLVX Tight Puro vector contains a restriction site for each of these enzymes. PCR products were fractionated on a 0.8 % (w/v) agarose gel (Figure 5.1). Lanes 1 - 10 all contain amplified dBp4 (847 bp); bands were excised and purified using the GeneJet purification kit.

Following purification of amplified dBp4, dBp4 and pLVX Tight Puro were digested with *BamH* and *EcoRI* restriction enzymes. Digested dBp4 was fractionated on a 0.8 % (w/v) agarose gel (Figure 5.2). Double digests were carried out on dBp4 (lane 2) and pLVX Tight Puro vector (Lane 3). Single digests were carried out using *BamH* and *EcoRI* on dBp4 (lane 4 & 5, respectively) or pLVX Tight Puro (lane 7 & 8, respectively). Single digests were carried out to demonstrate that each restriction enzyme was working efficiently. Linearised plasmid DNA migrates more slowly through the gel than supercoiled plasmid DNA. Lane 8 shows pLVX Tight Puro vector digested with *BamH* and *EcoRI* and undigested vector is shown in lane 9. As both enzymes effectively digested pLVX Tight Puro, the double digest of the dBp4 construct should also be complete. *BamH* and *EcoRI* digested insert and vector bands were excised and purified using the GeneJet purification kit.

Gel purified *BamH* and *EcoRI* digested dBp4 was ligated into *BamH* and *EcoRI* digested pLVX Tight Puro vector. Ligation controls were set up; vector with no insert, vector and insert with no ligase, single digested vector with or without ligase to assess self-ligation and that ligase was working. One Shot® TOP10 competent *E.coli* were transformed with ligations. Once ampicillin resistant colonies were established, 28 colonies were picked and streaked onto a fresh agar plate and a portion added to Triton X-100 (5 % v/v) and heated to 95 °C to release DNA. PCR amplification with dBp4-specific primers (2.4.4) was carried out to screen for the insert (dBp4) in the bacterial colonies. PCR products were fractionated on 0.8 % (w/v) agarose gel (Figure 5.3 A & B). pTriEx4 Neo dBp4 (+) was used as a positive control for dBp4 PCR (Figure 5.3 A, (+)), with the correct band size seen at 827 bp. Two bands were seen in the positive control. This may have been due to coiled pTriEx4 Neo plasmid. The negative control contained no DNA (-) and no PCR product was generated (Figure 5.3 A, (-)). A band at 827 bp was amplified from colonies 23, 24 and 28 (Figure 5.2 B, lane 11, 12 and 14, respectively). Colony PCR is a crude method for determining the presence of the selected insert and non-specific amplification was seen in many of the colonies; this may be due to the composition of the primers which required a low annealing temperature (50 °C) and the quality of DNA isolated by this method is "quick and dirty".
Figure 5.1: Addition of BamHI and EcoRI restriction sites to dBp4 insert. Recognition sites for BamHI (5' end) and EcoRI (3' end) were added to dBp4 using dBp4-specific PCR primers with restriction enzyme sites at the 5'end of forward (BamHI) and reverse (EcoRI) primers. PCR products (847 bp) were fractionated on a 0.8 % (w/v) agarose gel (lanes 1 - 10), were excised and purified using the GeneJet gel purification kit: Position of the molecular weight marker is indicated (bp).
Figure 5.2: Restriction digest of dBp4 insert and pLVX Tight Puro vector. Plasmids and digested products were fractionated on a 0.8% (w/v) agarose gel. Lane 1 shows 10 kB DNA ladder, lane 2 shows BamH\text{I} and EcoRI digested dBp4, lane 3 shows BamH\text{I} and EcoRI digested vector (pLVX Tight Puro), lane 4 shows BamH\text{I} digested insert, lane 5 shows EcoRI digested insert, lane 6 shows undigested insert, lane 7 shows BamH\text{I} digested vector (pLVX Tight Puro), lane 8 shows EcoRI digested vector and lane 9 shows undigested vector. Position of molecular weight marker is indicated (bp).
Figure 5.3 A & B: Screening for dBP4 insert by colony PCR. Following transformation of One Shot® TOP10 E.coli with pLVX Tight Puro dBP4 ligations, colonies were picked onto a fresh agar replica plate and dBP4-specific PCR was carried out on 28 colonies. PCR products were fractionated on a 0.8 % (w/v) agarose gel. 1 ng pTriEx4 Neo dBP4 (+) was amplified as a positive control (A, (+)) and the negative control (-) contained no template DNA (A, (-)). Clone number is indicated above each lane and position of molecular weight marker is indicated (bp).
DNA was isolated from colonies 23, 24 and 28 and was sent for sequencing (LGC Genomics, Germany) to confirm the orientation of the insert from each clone and to check that no mutations occurred within the insert. Clone 24 was found to contain the dBP4 in the correct orientation and was used to inoculate 250 ml LB broth. Clone 23 and 28 did not contain the full-length dBP4 insert. pLVX Tight Puro dBP4 plasmid was purified from clone 24 grown in LBAmp broth using the Qiagen Endofree Maxi Prep system for transduction of PC-3M-luc2 cells. Endotoxin free plasmid was fractionated on 1% (w/v) agarose gel to ensure plasmid integrity (Figure 5.4).

5.2.2 Transduction of PC-3M-luc2 and HEK 293 cells with pTet-On Advanced vector

HEK 293 and PC-3M-luc2 cells were transduced with pTet-On Advanced vector, which produces rtTA that binds to the tetracycline response element (TRE mod) switching on dBP4, by use of the CMV promoter in the presence of doxycycline in the pLVX Tight Puro vector. The efficacy of the transduction was assessed by transfection of pTet-On HEK 293 cells with a reporter plasmid pLVX Tight Puro-luc that expressed luciferase in response to doxycycline or p16-luc, which expressed luciferase constitutively. The PC-3M-luc2 cells could not be tested in this way as they already express luciferase. A GFP plasmid was used as control for the transfection in HEK 293 cells. pTet-On HEK 293 cells were treated for 72 h with (+) or without (-) doxycycline (0.1 μM). Following addition of d-luciferin, cells were imaged using the IVIS imaging system, which detects bioluminescence (Figure 5.5). In Figure 5.5, wells 1 and 4 show pTet-On HEK 293 cells transfected with the inducible pLVX Tight Puro-luc plasmid. No bioluminescence was seen in the absence of doxycycline treatment (well 1), but with doxycycline, bioluminescence was observed (well 4). Bioluminescence in pTet-On HEK 293 cells transfected with a vector constitutively expressing luciferase (p16-luc, well 2 and 5) and treated with (well 5) or without (well 2) doxycycline was similar. In pTet-On HEK293 cells transfected with GFP-F8, no bioluminescence was detected with (well 6) or without doxycycline (well 3). This confirmed that pTet-On HEK 293 cells were transduced with pTet-On Advanced plasmid and from this we assumed that PC-3M-luc2 cells were also transduced.

5.2.3 Expression of rtTA and dBP4 in doxycycline induced expression system

Expression of rtTA by PC-3M-luc2 cells was demonstrated by Western blot. Whole cell lysate from pTet-On PC-3M-luc2 cells and PC-3M-luc2 (negative control) were fractionated by 4 - 20% (w/v) SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-rtTA and anti-β-actin antibodies. The predicted molecular weight of rtTA is 30 kDa. Figure 5.6 A shows that rtTA is expressed by PC-3M-luc2 cells transduced with pTet-On Advanced
Figure 5.4: Purified pLVX Tight Puro dBp4 plasmid. Plasmid DNA was purified from clone 24 using Qiagen endotoxin free maxi prep and fractionated on a 1 % (w/v) agarose gel. Lane 1 shows 10 kB DNA ladder and lane 2 shows endotoxin free maxi prep pLVX Tight Puro dBp4 plasmid (1 μg). Position of molecular weight marker is indicated (bp).
Figure 5.5: Doxycycline induced luciferase activity in pTet-On Advanced HEK 293 cells. HEK 293 cells transduced with the pTet-On vector were transfected with either pLVX Tight Puro-luc (wells 1 and 4, doxycycline inducible luciferase), p16-luc (wells 2 and 5, constitutively expressed luciferase) or GFP-F8 (wells 3 and 6, negative control) and incubated with (+) or without (-) doxycycline (0.1 μM) for 72 h then imaged using the IVIS Spectrum.
vector (lane 1) and not expressed in untransduced PC-3M-luc2 cells (lane 2) were negative for rtTA. Following selection of stable pTet-On PC-3M-luc2 cells in G418 (400 µg/ml) for 4 weeks, cells were transduced with pLVX Tight Puro dBP4, then selected in puromycin (2 µg/ml) for 10 days, placed in serum free medium with or without doxycycline (0.1 µM) for 72 h. Whole cell lysate and conditioned medium were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and dBP4 expression was assessed using anti-His-tag antibody (Figure 5.6 B). The His-tag differentiates between exogenous dBP4 and endogenous wildtype IGFBP 4. Figure 5.6 B shows that His-tagged dBP4 was only expressed in the cell lysate (lane 2) and conditioned medium of PC-3M-luc2 dBP4 cells treated with doxycycline (lane 4). dBP4 was at a lower molecular weight in conditioned medium compared with cell lysate due to cleavage of the signal sequence on secretion of dBP4. dBP4 was not detected in cell lysate (lane 3) or conditioned medium (lane 5) from untreated PC-3M-luc2 dBP4 cells. Thus, the doxycycline inducible system is under tight control and does not ‘leak’ in the absence of doxycycline.

5.3 Effect of inducible vector expressed dBP4 on prostate tumour growth in vivo

5.3.1 Expression dBP4 in a subcutaneous prostate cancer model

A pilot study was carried out to demonstrate doxycycline inducible dBP4 expression in vivo. PC-3M-luc2 cells transduced with pTet-On and pLVX Tight Puro dBP4 were implanted subcutaneously into the right flank of NSG mice (n=15). When tumours reached a mean tumour diameter (MTD) of 7 - 8 mm mice were randomised into 3 groups. dBP4 expression was induced by feeding chow containing doxycycline (200 mg/kg) (n=5). Controls were given normal chow (n=5). The IGF IR TKI, NVP-AEW541 (50 mg/kg) was administered daily by oral gavage (n=5). After 3 days or 7 days treatment, 2 mice from each group were sacrificed and IGF I levels were measured by ELISA on both serum and tumour tissue. Tumour IGF I was expressed as ng/mg of total tumour protein. As there was only n = 2 statistics could not be carried out (Figure 5.7 A & B). There was no difference in serum IGF I levels between the treatment groups at day 3 or day 7 (Figure 5.7 A). From day 3 - day 7 tumour IGF I levels increased in controls, but not in dBP4-expressing or NVP-AEW541-treated tumours. Tumour IGF I levels were higher in NVP-AEW541-treated tumours relative to controls or dBP4-expressing tumours. The effect of dBP4 or NVP-AEW541 on tumour growth was not monitored as the study was only carried out for 7 days. dBP4 expression in the tumours was assessed by Western blotting using anti-His-tag antibody. Tumour lysates (50 µg) were fractionated by 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and dBP4 expression was assessed by anti-His-tag antibody (Figure 5.8). The anti His-tag antibody allows for identification of vector-expressed dBP4 vs. wildtype IGFBP 4.
Figure 5.6 A & B: rtTA and dBP4 are expressed by PC-3M-luc2 cells. PC-3M-luc2 cells were transduced with pTet-On plasmid which produces the regulatory protein, reverse tetracycline-controlled transactivator (rtTA). Cell lysates from PC-3M-luc2 cells transduced with pTet-On vector (A, lane 1) and untransduced cells (A, lane 2) were fractionated on 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and probed with an antibody to the Tet induced rtTA. β-actin was used as a loading control. Following selection of transduced cells in G418, the pTet-On PC-3M-luc2 cells were transduced with the response plasmid (pLVX Tight Puro dBP4) containing dBP4. Western blot carried out on the PC-3M-luc2 dBP4 cells treated with or without doxycycline (0.1 μM doxycycline, B) for 72 h. Cell lysate and conditioned medium were fractionated on 4 - 20 % (w/v) SDS-PAGE, transferred to nitrocellulose and probed with anti His-tag antibody (B). Lane 1 shows positive control (his-tagged protein ladder), lane 2 shows cell lysate from doxycycline treated PC-3M-luc2 dBP4 cells, lane 3 shows cell lysate from untreated PC-3M-luc2 dBP4 cells, lane 4 shows conditioned medium from doxycycline treated PC-3M-luc2 dBP4 cells and lane 5 shows conditioned medium from untreated PC-3M-luc2 dBP4 cells. Position of molecular weight marker is indicated (kDa).
Following addition of doxycycline chow to dB4 mice, dB4 expression was induced at day 3 (lanes 3 & 9) and remained expressed at day 7 (lanes 4 & 10). Controls or NVP-AEW541 treated tumours showed low levels of dB4 expression (lanes 8, 11 and 12 respectively).

5.3.2 Long-term effect of inducible vector expressed dB4 in a subcutaneous model of prostate cancer

Following a pilot study showing that doxycycline-induced dB4 expression in the subcutaneously implanted PC-3M-luc2 dB4 tumours in vivo, PC-3M-luc2 dB4 cells (5 x 10^6 cells/100 μl) were implanted subcutaneously in male NSG mice (n=24). Tumour growth was monitored by caliper measurement every 3 - 4 days. When tumours reached a MTD of 7 - 8 mm mice were randomised into 3 groups. dB4 expression was induced by feeding chow containing doxycycline (200 mg/kg) (n=8). Controls were given normal chow (n=8). The IGF IR TKI, NVP-AEW541 (50 mg/kg) was administered daily by oral gavage (n=8). Along with caliper measurement, tumour growth was monitored by bioluminescent imaging as the PC-3M-luc2 cells express luciferase, upon injection of luciferin light is emitted from the PC-3M-luc2 cells, which is then detected by the IVIS imaging system. Mice received an i.p. injection of luciferin (75 mg/kg) and were imaged 5 min later while kept under 2% (v/v) isoflurane. Imaging was carried out every 3 - 4 days up to day 20 of treatment (Figure 5.9). Following day 20 tumours began to become visibly necrotic therefore BLI was not used thereafter as luciferase is only expressed in viable cells and BLI would therefore under estimate tumour burden. In addition, BLI was not used in large subcutaneous tumours because the superficial location results in saturation of signal and non-linear correlation with tumour size.

Figure 5.9 shows IVIS images from controls, dB4-expressing or NVP-AEW541-treated PC-3M-luc2 dB4 subcutaneous tumour bearing mice. Controls showed an increase in tumour growth and BLI by treatment day 20. dB4-expressing tumours also showed an increase in tumour growth and BLI. NVP-AEW541-treated tumours showed a rapid increase in tumour growth and BLI. Using Living Image 3.2 software, bioluminescence (ph/s/cm²/sr) was calculated for each tumour within all treatment groups. Photons are a measurement of radiance on the surface of the mouse. This measurement automatically takes into account the camera settings. Images of subjects acquired in the same session have the same signal amplitude as the radiance of the animal surface does not change. Therefore, this allows direct comparison of mice imaged throughout the study. No significant difference in bioluminescence was seen between each treatment group (Figure 5.10 A). However, from treatment day 14 - day 20 dB4-expressing tumours were significantly smaller in MTD relative to controls (10.1 ± 0.27 mm vs. 11 ± 0.28 mm, p<0.05 (day 14),
Figure 5.7 A & B: Serum and tumour IGF I levels in mice with subcutaneously implanted PC-3M-luc2 dBp4 cells. Serum and tumours were collected from NSG mice implanted with PC-3M-luc2 dBp4 subcutaneous tumours following treatment with normal chow (controls, 3 days (n=2/group) or 7 days (n=2/group)), doxycycline chow (200 mg/kg, 3 days (n=2/group) or 7 days (n=2/group)) or NVP-AEW541 (50 mg/kg, 3 days (n=2/group) or 7 days (n=2/group)). IGF I levels were measured in duplicate by ELISA in serum (A) and tumour lysates (B). Total tumour protein was assessed by Bio-Rad DC protein assay. Tumour IGF I was expressed as ng IGF I/mg total protein. Data (n=2 mice/group) are expressed as mean.
His-tagged dBP4
(34 kDa)

Figure 5.8: dBP4 is expressed in subcutaneously implanted PC-3M-luc2 dBP4 cells following doxycycline treatment. $5 \times 10^6$ PC-3M-luc2 dBP4 cells were implanted subcutaneously into the right flank of NSG mice ($n=15$). Following treatment of NSG mice with normal chow (controls), doxycycline chow (200 mg/kg) or NVP-AEW541 (50 mg/kg daily by oral gavage), two mice from each group were sacrificed after day 3 and day 7 to test for dBP4 expression. Tumour lysates (50 µg) were fractionated by 4 - 20 % SDS-PAGE, transferred to nitrocellulose and probed with anti-His-tag antibody. Lanes 1 and 2 show tumour lysates from control mice at day 3 and day 7, respectively. Lanes 3 and 4 show tumour lysates from doxycycline treated mice at day 3 and day 7, respectively. Lanes 5 and 6 show tumour lysates from NVP-AEW541 treated mice at day 3 and day 7, respectively. Lanes 7 and 8 show tumour lysates from control mice at day 3 and day 7, respectively. Lane 9 and 10 show tumour lysates from doxycycline treated mice at day 3 and day 7, respectively. Lanes 11 and 12 show tumour lysates from NVP-AEW541 treated mice at day 3 and day 7, respectively. Position of molecular weight marker is indicated (kDa).
Figure 5.9: Bioluminescent imaging of PC-3M-luc2 dBP4 implanted subcutaneous tumours expressing dBP4 or treated with NVP-AEW541. PC-3M-luc2 dBP4 cells (5 x 10^6 cells) were implanted subcutaneously into the right flank of NSG mice (n=24). When tumours reached 7 - 8 mm MTD mice were randomised into groups. Controls received normal chow, dBP4 mice received doxycycline chow (200 mg/kg) or NVP-AEW541 (50 mg/kg, daily by oral gavage) (n=8 mice/group). Tumour growth was monitored every 3 - 4 days by IVIS imaging and caliper measurements. Day 0 represents 16 days post implantation of PC-3M-luc2 dBP4 cells before treatment and treatment was started on day 1 (23 days post PC-3M-luc2 dBP4 cell implantation). Mice received an i.p. injection of luciferin (75 mg/kg) and were imaged 5 min later under 2% (v/v) isoflurane. Mice were imaged for 1 s at f stop 1 from day 0 - day 20 of treatment.
11 ± 0.41 mm vs. 12.22 ± 0.38 mm, p<0.041 (day 18) and 11.8 ± 0.41 mm vs. 13.1 ± 0.49 mm, p<0.05 (day 20)) or NVP-AEW541-treated tumours (10.1 ± 0.27 mm vs. 11.5 ± 0.41 mm, p<0.008 (day 14), 11 ± 0.41 mm vs. 13.3 ± 0.39 mm, p<0.0001 (day 18) and 11.8 ± 0.41 mm vs. 14.2 ± 0.96 mm, p<0.001 (day 20)) (Figure 5.10 B). NVP-AEW541 did not inhibit tumour growth in this experiment. The MTD further supports the IVIS images where dBP4-expressing tumours were smaller than controls or NVP-AEW541-treated tumours, but the average bioluminescence for each group did not reflect that of the MTD, which may have been attributed to necrotic regions in larger tumours. Percent survival as length of time to sacrifice was calculated for all groups. In this experiment, mice were sacrificed when tumours reached a MTD of 20 mm or when mice began to show signs of discomfort. Assessing the mice for illness was carried out blind in order to alleviate bias based on treatment groups. The majority of mice did not reach 20 mm MTD due to discomfort or leg paralysis. The Kaplan-Meier curve shows mice bearing dBP4-expressing tumours survived longer than controls (37 ± 2.4 days vs. 27.8 ± 2.4 days, p<0.04) or NVP-AEW541-treated mice (37 ± 2.4 days vs. 27.3 ± 2.1 days, p<0.04) (Figure 5.11).

The main side effect reported with other strategies or inhibitors of the IGF pathway is hyperglycaemia (Crouthamel et al., 2009). For this reason, blood glucose was measured from non-fasting mice before treatment initiation (pre-treatment) and upon sacrifice of mice (post-treatment). Mice were not fasted as hyperglycaemia should be apparent as a glucose reading >200 mg/dL or 11 mmol/L (Kobayashi et al., 2003) and IGF I levels decrease upon fasting, which could interfere with the study (Frystyk et al., 1999). Blood glucose was measured using AlphaTRAK glucose meter. A paired t-test was carried out to compare pre-treatment glucose levels to post-treatment levels in all 3 groups. Post-treatment glucose levels were significantly higher than pre-treatment glucose levels in mice bearing subcutaneous dBP4-expressing tumours, (8.08 ± 0.43 mmol/L vs. 9.95 ± 0.9 mmol/L, p<0.019). Controls (7.7 ± 0.3 mmol/L vs. 9.55 ± 0.8 mmol/L) or NVP-AEW541-treated mice (7.67 ± 0.4 mmol/L vs. 9.71 ± 0.7 mmol/L) showed no significant change in glucose levels post-treatment (Figure 5.12). As NVP-AEW541 treatment showed no effect on tumour growth, a change in glucose was not expected. As IGFBP 4 inhibits IGF I, IGF I levels were measured in duplicate by ELISA in serum and subcutaneous tumours. The majority of IGF I within the body is bound to IGFBPs (Poffenbarger et al., 1968), therefore IGF I bound to IGFBPs and unbound IGF I (total IGF I) was measured. Tumour IGF I was measured as ng/mg total tumour protein. Serum IGF I was increased in mice with subcutaneous dBP4-expressing tumours relative to controls (808.4 ± 23 ng/ml vs. 647.8 ± 53.8 ng/ml, p<0.033) or NVP-AEW541-treated mice (808.4 ± 23 ng/ml vs. 635.4 ± 65.5 ng/ml, p<0.018) (Figure 5.13 A).
Figure 5.10 A & B: Inducible vector expressed dBP4 reduces growth of subcutaneously implanted PC-3M-luc2 cells. PC-3M-luc2 dBP4 (5 x 10^6 cells) cells were implanted subcutaneously into the right flank of NSG mice (n=24). Mice were randomised into groups when tumours reached a MTD of 7 - 8 mm. Controls received normal chow, dBP4 mice received doxycycline chow (200 mg/kg) or NVP-AEW541 (50 mg/kg, daily by oral gavage) (n=8 mice/group). Tumour growth was monitored every 3 - 4 days by IVIS imaging (A) and caliper measurements (B). Luciferin was delivered i.p. at 75 mg/kg and mice were imaged 5 min later while kept under 2% (v/v) isoflurane. Living Image 3.2 software was used to calculate the bioluminescence of each individual subcutaneous tumour. Data (n=8 mice/group) is expressed as mean ± SEM. Data was analysed by One way ANOVA followed by LSD post hoc correction test. *p<0.05 vs. Control, $$p<0.01 vs. dBP4, $$$p<0.001 vs. dBP4.
Figure 5.11: Inducible vector expressed dBp4 increases survival of mice with PC-3M-luc2 dBp4 implanted subcutaneous tumours. Kaplan-Meier plot showing increased time to sacrifice in NSG mice bearing PC-3M-luc2 dBp4 implanted subcutaneous tumours expressing dBp4 (doxycycline chow, n=8) relative to controls fed normal chow (n=8) and NVP-AEW541 treated mice (50 mg/kg, daily by oral gavage, n=8) ($\chi^2 = 6.17$, $p<0.05$). Time to sacrifice was based on tumours reaching a MTD of 20 mm or signs of impaired mobility (leg paralysis) and discomfort.
Figure 5.12: Non-fasting blood glucose levels increased post-treatment in mice bearing dBP4-expressing subcutaneous tumours. Blood glucose was measured in controls fed normal chow (n=8), dBP4-expressing mice fed doxycycline chow (200 mg/kg) n=8) or NVP-AEW541-treated (50 mg/kg, daily by oral gavage, n=8) mice using AlphaTRAK glucose meter. Pre-treatment and post-treatment blood glucose was measured from the lateral tail vein and upon sacrifice of mice by cardiac puncture, respectively. Data (n=8 mice/group) are expressed as mean ± SEM. A paired t-test was carried out to test for differences between pre-treatment glucose and post-treatment glucose for each group (*p<0.05).
However, tumour IGF I was decreased in dBPs-expressing tumours relative to controls (0.6 ± 0.05 ng/mg vs. 0.79 ± .07 ng/mg; p<0.024) or NVP-AEW541-treated tumours (0.6 ± 0.05 ng/mg vs. 0.93 ± 0.03 ng/mg, p<0.012) (Figure 5.13 B). This suggests that dBPs is having an effect locally. Subcutaneous tumours were lysed and 4 tumours each from controls, dBPs-expressing tumours or NVP-AEW541-treated tumours were assessed for expression of dBPs and components of the IGF I signalling pathway including IGF IRβ, pIGF IR, Akt, pAkt, ERK and pERK. 50 μg of protein was fractionated by 4 - 20 % SDS-PAGE, transferred to nitrocellulose and probed for the aforementioned proteins. dBPs was not expressed in control tumour lysates (lanes 1 - 4). In lanes 5 - 8 dBPs remained expressed in 3 out of 4 doxycycline treated tumours up to time of sacrifice (32 - 34 days). NVP-AEW541-treated tumours showed no dBPs expression (lanes 9 - 12) (Figure 5.14 A). As IGF I activates the PI3K and MAPK pathways (Valentinis et al., 1999), activation of both of these pathways was assessed in tumour tissue following dBPs induction and treatment with NVP-AEW541. In 3 out of 4 dBPs-expressing tumours (lanes 5 - 7) a decrease in IGF IR activation (pIGFR) was seen relative to controls (lanes 1 - 4) (Figure 5.14 B). The tumour shown in lane 8 did not express dBPs despite doxycycline treatment and pIGFR was not reduced. However, no change in pIGFR was seen in NVP-AEW541-treated tumours (lanes 9 - 12) relative to controls indicating that NVP-AEW541-(50 mg/kg) was inactive or ineffective at the dose used. α-tubulin was lower in lanes 3 - 4, however a decrease in phospho Akt (Ser 473) was seen between controls (lanes 1 and 2) and dBPs-expressing tumours (lanes 5 - 7). Phospho Akt (Ser 473) was higher in 3 out of the 4 NVP-AEW541-treated tumours (lanes 9 - 11) relative to controls or dBPs-expressing subcutaneous tumours. The loading control α-tubulin shows unequal loading. However, densitometry was carried in order to normalise protein bands to α-tubulin (data not shown). Total Akt levels did not change in all treatment groups. No change in phospho ERK (Thr 202/Tyr204) was reported in dBPs-expressing tumours (lanes 5-8) relative to controls (lanes 1 - 4). However, phospho ERK (Thr 202/Tyr204) was decreased in NVP-AEW541 treated tumours (lanes 9 - 12) relative to controls (lanes 1 - 4) or dBPs-expressing tumours (lanes 5 - 8) although NVP-AEW541 had no effect on growth or survival or IGF IR activation (Figure 5.10 and Figure 5.11, respectively). Total ERK did not change in all 3 groups (Figure 5.14 B).

The effect of dBPs on the angiogenic factor, VEGF was assessed by Western blot. The antibody detects the VEGF isoforms, VEGF121 (15 - 18 kDa), VEGF165 (24 kDa) and VEGF189 (28 - 30 kDa). However, VEGF may also be produced from host cells (mouse). Therefore, VEGF isoforms, VEGF120 (14 kDa), VEGF164 (23 kDa) and VEGF188 (27 kDa) may also be present. VEGF164/165 was expressed in 3 of 4 control tumours (lanes 1 - 4), but was not expressed in the dBPs-expressing tumours (lanes 5 - 8) or NVP-AEW541-treated tumours (lanes 9 - 12).
Figure 5.13 A & B: Serum IGF I increased in NSG mice bearing dBP4-expressing subcutaneous tumours whereas IGF I was decreased in dBP4-expressing subcutaneous tumours. Serum and tumours were collected from NSG mice with PC-3M-luc2 dBP4 implanted subcutaneous tumours treated with doxycycline chow (200 mg/kg, n=8), controls fed normal chow (n=8) or NVP-AEW541-treated (50 mg/kg daily by oral gavage, n=8). IGF I levels were measured in duplicate by ELISA in serum (A) and tumour lysates (B). Total tumour protein was assessed by Bio-Rad DC protein assay. Tumour IGF I was expressed as ng IGF I/mg total protein. Data (n=8 mice/group) are expressed as mean ± SEM. Data was analysed by one-way ANOVA followed by LSD post hoc correction (*p<0.05 dBP4 vs. Control).
Two of 4 NVP-AEW541-treated tumours expressed VEGF_{188/189} (lanes 9 - 12) whereas neither controls (lanes 1 - 4) nor dBP4-expressing tumours (lanes 5 - 8) expressed this isoform. VEGF_{120/121} was not expressed by any of the treatment groups (Figure 5.14 C).

5.3.3 Effect of inducible vector expressed dBP4 in an orthotopic model of prostate cancer

Tumour microenvironment is important in tumour growth and development. To represent human prostate cancer PC-3M-luc2 dBP4 (1 × 10^5 cells / 50 pl) cells were implanted into the prostate of NSG mice (n=20). This study was run in parallel to the subcutaneous prostate cancer model. Tumour growth was monitored by IVIS imaging every 3 - 4 days. Mice received an i.p. injection of luciferin (75 mg/kg) and were imaged 5 min later while kept under 2% (v/v) isoflurane. Once tumours were established mice were randomised into 3 groups. dBP4 expression was induced by feeding chow containing doxycycline (200 mg/kg) (n=7). Controls were given normal chow (n=7). The IGF IR TKI, NVP-AEW541 (50 mg/kg) was administered daily by oral gavage (n=6). IVIS images of prostate tumours up to treatment day 19 are shown (Figure 5.15). Control prostate tumours show an increase in tumour size and BLI. dBP4-expressing prostate tumours did not increase significantly in size over the 19 days and BLI is lower compared with controls. Two control mice and dBP4-expressing mice were sacrificed before day 19. NVP-AEW541-treated tumours grew rapidly from day 4 - day 19, one mouse treated with NVP-AEW541 was sacrificed before day 14 and 2 mice were sacrificed before day 19. Bioluminescence was measured for each prostate tumour within each group by Living Image 3.2. software. Readings were measured as photons/sec. At day 19 of treatment dBP4-expressing prostate tumours showed a significant decrease in bioluminescence relative to controls (2.74 x 10^9 ± 6.9 10^6 p/s/cm^2/sr vs. 48 x 10^9 ± 1.2 x 10^9 p/s/cm^2/sr, p<0.0097) (Figure 5.16). Only 2 mice were left in the NVP-AEW541 group at day 19, therefore they were not included in the statistical analysis. BLI was not used after day 19 as mice were too sick to anaesthetise and were euthanised. Percent survival as length of time to sacrifice was calculated for all groups. In this experiment mice were sacrificed when a sign of illness including depressed activity, unkept haircoat, hunched posture or decreased food/water intake was noted. Figure 5.17 shows the Kaplan Meier plot, neither dBP4 expression nor NVP-AEW541 treatment did not significantly increase survival in prostate tumour bearing mice relative to controls (22.3 ± 3.4 days vs. 18.8 ± 1.9 days, 18.5 ± 3.7 days vs. 18.8 ± 1.9 days, respectively). This conflicts with the BLI data where dBP4-expressing tumours showed decreased bioluminescence, an increase in survival may not have been seen due to the location of the tumours as the prostate tumours may have impinged on other organs.

154
Figure 5.14 A, B & C: dBP4 is expressed in PC-3M-luc2 dBP4 implanted subcutaneous tumours and inhibits IGF IR activation. Subcutaneous tumours were collected from control (normal chow, n=8), dBP4-expressing (doxycycline chow, n=8) or NVP-AEW541-treated (50 mg/kg, daily by oral gavage, n=8) mice (32 - 34 days post treatment) and snap frozen. Tumour lysates were made from 4 tumours from each group. 50 µg protein was fractionated by 4 - 20 % SDS-PAGE, transferred to nitrocellulose and probed with the above antibodies. In A, B & C, lanes 1 - 4 show tumour lysates from control mice, lanes 5 - 8 show tumour lysates from dBP4-expressing mice and lanes 9 - 12 show tumour lysates from NVP-AEW541-treated mice. VEGF expression may have originated from either PC-3M-luc2 cells (human) or host cells (mouse). Human and mouse VEGF isoforms differ in size. The human isoforms are VEGF121 (15 - 18 kDa), VEGF165 (24 kDa), VEGF189 (28 kDa). The mouse isoforms are VEGF120 (14 kDa), VEGF164 (23 kDa) and VEGF188 (27 kDa). Blots were probed with anti-α tubulin as a loading control. Position of molecular weight marker is indicated (kDa).
Figure 5.15: Bioluminescent imaging of PC-3M-luc2 dBp4 prostate tumours expressing dBp4 or treated with NVP-AEW541. PC-3M-luc2 dBp4 (1 \times 10^5 cells) cells were implanted into the prostate of NSG mice (n=20). Mice were randomised into groups. Controls received normal chow (n=7), dBp4 mice received doxycycline chow (200 mg/kg) (n=7) or NVP-AEW541 (50 mg/kg), given daily by oral gavage (n=6). Tumour growth was monitored every 3 - 4 days by IVIS imaging. Day 0 represents 16 days post implantation of PC-3M-luc2 dBp4 cells before treatment and treatment was initiated on day 1 (22 days post implantation of PC-3M-luc2 dBp4 cells). Mice received an i.p. injection of luciferin (75 mg/kg) and were imaged 5 min later while kept under 2% (v/v) isoflurane. Mice were imaged for 1 s at f-stop 1 from day 0 - day 19.
Figure 5.16: dBP4-expressing prostate tumours show a decrease in BLI compared to controls and NVP-AEW541 treated tumours. PC-3M-luc2 dBP4 (1 x 10^5 cells) cells were implanted into the prostate of NSG mice (n = 20). Mice were randomised into groups; controls received normal chow (n=7), dBP4 mice received doxycycline chow (200 mg/kg, n=7) and IGF IR TKI, NVP-AEW541 (50 mg/kg, n=6), was given daily by oral gavage. Prostate tumour growth was monitored every 3 - 4 days by IVIS imaging. Luciferin was delivered i.p. at 75 mg/kg and mice were imaged 5 min later while kept under 2% (v/v) isoflurane. Living Image 3.2 software was used to calculate the bioluminescence of each individual prostate tumour. Data (n=6/7 mice/group) are expressed as mean ± SEM. Data was analysed by student t-test to compare dBP4 to controls at day 19 (**p<0.01). dBP4 vs. Control.
Figure 5.17: Inducible vector expressed dBp4 or an IGF IR TKI, NVP-AEW541 had no effect on survival in mice bearing PC-3M-luc2 dBp4 prostate tumours. Kaplan-Meier plot showing percent survival (time to sacrifice) in mice with PC-3M-luc2 dBp4 prostate tumours. Controls fed normal chow (n=7), dBp4-expressing mice fed doxycycline chow (n=7), or NVP-AEW541 treated (50 mg/kg given daily by oral gavage, n=6) mice. Time to sacrifice was based on signs of illness including depressed activity, unkept haircoat, hunched posture or decreased food/water intake (p = ns).
Non-fasting blood glucose levels were measured using the AlphaTRAK glucose meter before treatment initiation (pre-treatment) and before mice were sacrificed (post-treatment). A paired t-test was carried out to compare pre-treatment glucose to post-treatment glucose levels in all groups. Control mice showed a significant decrease in post-treatment glucose compared to pre-treatment glucose levels (7.28 ± 0.3 mmol/L vs. 4.86 ± 0.29 mmol/L, p<0.003). Decrease in blood glucose may have been due to hypoglycaemic effect seen in tumour bearing mice (Takeda et al., 1984) as controls were below 5 mmol/L (4.86 mmol/L). Glucose levels were unchanged post-treatment in the dBP4-expressing mice (8.25 ± 0.6 mmol/L vs. 7.11 ± 1 mmol/L) or NVP-AEW541-treated mice (7.98 ± 0.4 mmol/L vs. 7.6 ± 0.7 mmol/L) (Figure 5.18). IGF I levels were measured in serum and prostate tumours in duplicate by ELISA. Tumour IGF I was measured as ng/mg of total tumour protein. There was no significant difference in serum IGF I levels in mice bearing dBP4-expressing tumours relative to controls (670.4 ± 104.4 ng/ml vs. 614.2 ± 70.8 ng/ml) or NVP-AEW541-treated mice (670.4 ± 104.4 ng/ml vs. 658.2 ± 64.1 ng/ml) (Figure 5.19 A). However, a significant decrease in IGF I was seen in dBP4-expressing prostate tumours relative to controls (0.52 ± 0.07 ng/mg vs. 0.83 ± 0.07 ng/mg, p<0.028). No significant difference in tumour IGF I was seen between NVP-AEW541 and controls (0.71 ± 0.2 ng/mg vs. 0.83 ± 0.07 ng/mg) or dBP4-expressing tumours (0.71± 0.2 ng/mg vs. 0.52 ± 0.07 ng/mg) (Figure 5.19 B).

Prostate tumours were harvested from mice in each treatment group; controls, dBP4-expressing tumours and NVP-AEW541 treated tumours. Prostate tumours were lysed, 3 tumours each from controls, dBP4-expressing mice or NVP-AEW541 treated mice were assessed for expression of dBP4 and components of the IGF I signalling pathway including IGF IRβ, pIGF IR, Akt, pAkt, ERK and pERK. 50 µg of protein was fractionated by 4 - 20 % SDS-PAGE, transferred to nitrocellulose and probed for the aforementioned proteins. dBP4 was not expressed in tumours from controls (lanes 1 - 3) or NVP-AEW541 treated mice (lanes 7 - 9). dBP4 was expressed in all 3 tumours from mice fed doxycycline chow (lanes 4 - 6) up to the time of sacrifice (~ 20 days of treatment) (Figure 5.20 A). Activation of the Akt and MAPK pathways were assessed in prostate tumour tissue following treatment with dBP4 or NVP-AEW541 (Figure 5.20 B). Two of the 3 dBP4-expressing tumours (lanes 5 - 6) showed a decrease in IGF IR activation (pIGFR) relative to controls (lanes 1 - 3). NVP-AEW541-treated tumours showed a decrease in pIGFR (lanes 7 - 9) relative to controls or dBP4-expressing tumours. Phospho Akt (Ser 473, pAkt) was higher in dBP4-expressing tumours (lanes 4 - 6) relative to controls (lanes 1 - 3) or NVP-AEW541-treated tumours (lanes 7 - 9). The loading control α-tubulin was higher in dBP4-expressing tumours. However, densitometry was carried in order to normalise protein bands to α-tubulin (data not shown). One of the 3 NVP-AEW541-treated tumours showed a decrease in phospho Akt.
Figure 5.18: Non-fasting blood levels decreased in orthotopic prostate cancer controls. Blood glucose levels were measured in controls fed normal chow (n=7), dBp4-expressing mice fed doxycycline chow (200 mg/kg, n=7) or NVP-AEW541-treated (50 mg/kg, daily by oral gavage, n=6) mice using AlphaTRAK glucose meter. Pre-treatment and post-treatment blood glucose was measured from the lateral tail vein and upon sacrifice of mice by cardiac puncture, respectively. Data (n=6/7 mice/group) are expressed as mean ± SEM. A paired t-test was carried out to test for differences between pre-treatment glucose and post-treatment glucose levels for each group (**p<0.01).
Figure 5.19 A & B: IGF I levels decreased in dB44-expressing prostate tumours. Serum and tumours were collected from NSG mice (n=20) with PC-3M-luc2 dB44 prostate tumours treated with doxycycline chow (200 mg/kg, n=7), controls fed normal chow (n=7) or NVP-AEW541-treated (50 mg/kg given daily by oral gavage, n=6). IGF I levels were measured in duplicate by ELISA in serum (A) and tumour lysates (B). Total tumour protein was assessed by Bio-Rad DC protein assay. Tumour IGF I was expressed as ng IGF I/mg total protein. Data (n=6/7 mice/group) are expressed as mean ± SEM. Data was analysed by one-way ANOVA followed by LSD post hoc correction (*p<0.05) dB44 vs. Control.
(pAkt, lane 8). Total Akt levels did not change in all groups. No difference in phospho ERK (Thr 202/Tyr204) was seen between all groups. In all groups no change in total ERK was seen (Figure 5.20 B). NVP-AEW541 had no effect on tumour growth or IGF signalling suggesting that it did not work in this study.

5.3.4 Effect of inducible vector expressed dBP4 in models of experimental metastasis
Bone is the most common site of metastases in prostate cancer patients (Bubendorf et al., 2000). In order to test the effect of dBP4 on metastasis formation two models were utilised. The intracardiac model involved injection of tumour cells into the left ventricle which gives rise to bone metastases and IV injection of tumour cells which leads to bone, lung and liver metastases. However, with the PC-3M-luc2 dBP4 cells few mice developed bone metastases in either model with the majority of metastases formed in the liver. The PC-3M cells are derived from a liver metastasis of parental PC-3 cells (Kozlowski et al., 1984).

5.3.4.1 Effect of inducible vector expressed dBP4 following intracardiac injection of PC-3M-luc2 dBP4 cells
PC-3M-luc2 dBP4 (5 x 10⁵ cells/100 µi) cells were injected into the left ventricle of NSG mice (n=12). Metastasis formation was monitored by IVIS imaging. Mice received an i.p. injection of luciferin (75 mg/kg) and were imaged 5 min later while kept under 2% (v/v) isoflurane. Once metastases were growing, mice with bioluminescent deposits (metastases) were randomised into 2 treatment groups. Six out of 12 mice developed metastases after 35 days. dBP4 expression was induced by feeding chow containing doxycycline (200 mg/kg) (n=3). Controls were given normal chow (n=3). Metastasis growth was monitored every 3 - 4 days. Mice were sacrificed once any sign of illness was seen. Figure 5.21, shows BLI images for controls and mice bearing dBP4-expressing liver metastases. Mice were monitored for 8 days by BLI as after day 8 bioluminescence decreased in both controls and dBP4-expressing mice suggesting that the metastases were becoming necrotic in both groups. Two of the control mice showed an increase in tumour burden by treatment day 8, with the majority of the liver containing tumour cells. One of the control mice showed a small deposit of tumour cells, which were not present by day 8. However, when mice were sacrificed all mice bore tumours. This suggests that the metastases in the control mouse may have become necrotic thus not detectable by BLI. dBP4-expressing liver metastases remained localised within the liver and did not diffuse throughout the liver relative to controls. However, dBP4-expressing mice showed a larger tumour burden on day 0 relative to controls. Percent survival as length of time to sacrifice was calculated for both groups. In this experiment mice were sacrificed when a sign of illness including depressed activity, unkept haircoat, hunched posture or decreased food/water intake was noted.

165
Figure 5.20 A & B: dBP4 is expressed in PC-3M-luc2 dBP4 prostate tumours and inhibits IGF IR activation. Prostate tumours were collected from controls fed normal chow (n=7), dBP4-expressing mice fed doxycycline chow (200 mg/kg, n=7) or NVP-AEW541-treated (50 mg/kg daily by oral gavage, n=6) mice (20 days post treatment) and snap frozen. Tumour lysates were made from 3 tumours from each group. 50 μg protein was fractionated by 4 - 20 % SDS-PAGE, transferred to nitrocellulose and probed with the above antibodies. In A & B, lanes 1 - 3 show tumour lysates from control mice, lanes 4 - 6 show tumour lysates from dBP4-expressing mice and lanes 7 - 9 show tumour lysates from NVP-AEW541 treated mice. Blots were probed with anti-α tubulin antibody as a loading control. Position of molecular weight marker is indicated (kDa).
Figure 5.21: Bioluminescent imaging of PC-3M-luc2 dBP4 cells following intracardiac injection and induction of dBP4. PC-3M-luc2 cells (5 × 10^4 cells) were injected via intracardiac injection in NSG mice (n=12). Mice were randomised into groups. Controls received normal chow and dBP4-expressing mice received doxycycline chow (200 mg/kg) (n=3 mice/group). Metastasis formation was monitored every 3 - 4 days by IVIS spectrum. Day 0 represents 35 days post injection of PC-3M-luc2 dBP4 cells before treatment and treatment was initiated on day 1 (42 days post injection of PC-3M-luc2 dBP4 cells). Mice received an i.p. injection of luciferin (75 mg/kg) and were imaged 5 min later while kept under 2% (v/v) isoflurane. Mice were imaged for 1 s at f stop 1 from day 0 - day 8.
Figure 5.22 shows the Kaplan Meier plot, no significant difference in survival was seen between controls and mice bearing dBP4-expressing liver metastases (19.7 ± 6.1 days vs. 12.5 ± 1.5 days) even though mice bearing dBP4-expressing liver metastases died sooner, the n numbers may have been too small to see a significant difference. The dBP4 expressing mice may have died sooner due to greater tumour burden at the beginning of the study.

Non-fasting glucose levels were measured using AlphaTRAK glucose meter before treatment initiation (pre-treatment) and before mice were sacrificed (post-treatment). A paired t-test was carried out to compare pre-treatment blood glucose to post-treatment levels in controls and mice bearing dBP4-expressing liver metastases (Figure 5.23). No significant change in glucose levels was seen upon sacrifice in controls (7.8 ± 0.05 mmol/L vs. 5.5 ± 0.6 mmol/L) or mice bearing dBP4-expressing liver metastases (7 ± 0.9 mmol/L vs. 6.6 ± 0.2 mmol/L). Serum IGF I levels were measured in duplicate by ELISA. There was no significant difference in serum IGF I levels between controls or mice bearing dBP4-expressing liver metastases (1092.7 ± 9.2 ng/ml vs. 941.3 ± 169 ng/ml) (Figure 5.24).

5.3.4.2 Effect of inducible vector expressed dBP4 on metastases following IV injection of PC-3M-luc2 cells

The second method for development of metastases is IV injection. PC-3M-luc2 dBP4 (2 x 10⁶ cells/250 µl) cells were injected into the lateral tail vein of NSG mice (n=20). Metastasis formation was monitored by IVIS imaging. Once it was established that metastases were growing, mice with bioluminescent deposits (metastases) were randomised into 2 treatment groups. After 43 days, 10 out of 20 mice developed metastases. dBP4 expression was induced by feeding chow containing doxycycline (200 mg/kg) (n=5). Controls were given normal chow (n=5). Mice were sacrificed once any sign of illness was seen. Figure 5.25, shows BLI images for controls and mice bearing dBP4 expressing liver metastases. Mice were monitored for 14 days by BLI as mice were not metabolising the luciferin after day 14 leading to abnormal bioluminescent readings, this may have been due to illness. One of the control mice showed a large increase in tumour burden by day 4, with the majority of the liver taken up with tumour cells at day 14 (Figure 5.25). Three of the 5 control mice and 2 of the 5 dBP4-expressing mice showed bone metastasis formation. However, BLI of the bone metastases fluctuated between each imaging session, therefore the effect of dBP4 on bone metastasis growth was not monitored. dBP4-expressing liver metastases remained localised within the liver (Figure 5.25). Bioluminescence was not seen in some of the mice on certain days of imaging, but upon sacrifice all mice showed tumours within the liver.
Figure 5.22: dBp4 had no effect on survival in liver metastasis bearing mice following intracardiac injection of PC-3M-luc2 dBp4 cells. Kaplan-Meier plot showing percent survival (time to sacrifice) in mice with liver metastases following intracardiac injection of PC-3M-luc2 dBp4 cells. Controls fed normal chow (n=3) and dBp4-expressing mice fed doxycycline chow (200 mg/kg, n=3). Time to sacrifice was based on signs of illness including depressed activity, unkept haircoat, hunched posture or decreased food/water intake (p = ns).
Figure 5.23: Non-fasting blood glucose levels are unaffected post-induction of dBP4. Blood glucose levels were measured in controls (normal chow, n=3) and dBP4 mice (doxycycline chow (200 mg/kg), n=3) using AlphaTRAK glucose meter. Pre-treatment and post-treatment blood glucose was measured from the lateral tail vein and upon sacrifice of mice by cardiac puncture, respectively. Data (n=3 mice/group) are expressed as mean ± SEM. A paired t-test was carried out to test for differences between pre-treatment glucose and post-treatment glucose levels for each group (p = ns).
Figure 5.24: Serum IGF I levels were unaffected following dBP4 expression compared with controls in liver metastases following intracardiac injection of PC-3M-luc2 dBP4 cells. Serum was collected from NSG mice with PC-3M-luc2 dBP4 liver metastases (intracardiac injection) treated with doxycycline chow (200 mg/kg, n=3) or controls fed normal chow (n=3). Serum IGF I levels were measured in duplicate by ELISA. Data (n=3 mice/group) is expressed as mean ± SEM. Data was analysed by student t-test (p = ns).
One control mouse was sacrificed before day 11 of treatment, another control mice and one mouse bearing dBP4-expressing liver metastases were sacrificed before day 14 of treatment. Percent survival as length of time to sacrifice was calculated for both groups. In this experiment mice were sacrificed when a sign of illness including depressed activity, unkept haircoat, hunched posture or decreased food/water intake was noted. Figure 5.26, the Kaplan Meier plot showed that no significant difference in survival was seen between controls and mice bearing dBP4-expressing liver metastases even though mice bearing dBP4-expressing liver metastases survived longer than controls (11.7 ± 5 days vs. 19.8 ± 5 days). The n numbers may have been too small (n=5/group) to see a significant difference.

Non-fasting glucose levels were measured using AlphaTRAK glucose meter before treatment initiation (pre-treatment) and before mice were sacrificed (post-treatment). A paired t-test was carried out to compare pre-treatment glucose levels to post-treatment levels in controls and mice bearing dBP4-expressing liver metastases. No significant change in glucose levels was seen post-treatment in controls (6.86 ± 0.7 mmol/L vs. 9.36 ± 0.3 mmol/L) or mice bearing dBP4-expressing liver metastases (7.41 ± 0.9 mmol/L vs. 9.5 ± 0.7 mmol/L) (Figure 5.27). Serum IGF I levels were measured in duplicate by ELISA. No significant difference in serum IGF I was seen between controls or mice bearing dBP4-expressing liver metastases (812.9 ± 52.2 ng/ml vs. 695.5 ± 52.8 ng/ml) (Figure 5.28).
Figure 5.25: Bioluminescent imaging of PC-3M-luc2 dBP4 cells following IV injection and induction of dBP4. PC-3M-luc2 cells (2 × 10^6 cells) were injected via IV injection (lateral tail vein) in NSG mice (n=20). Mice were randomised into treatment groups based on the presence of metastases. Controls received normal chow and dBP4-expressing mice received doxycycline chow (200 mg/kg) (n=5 mice/group). Day 0 represents 42 days post injection of PC-3M-luc2 dBP4 cells before treatment and treatment was initiated on day 1 (47 days post injection of PC-3M-luc2 dBP4 cells). Metastasis formation was monitored every 3 - 4 days by IVIS spectrum. Mice received an i.p. injection of luciferin (75 mg/kg) and were imaged 5 min later while kept under 2% (v/v) isoflurane. Mice were imaged for 1 s at f-stop 1 from day 0 - day 14.
Figure 5.26: dBP4 expression had no effect on survival in liver metastasis bearing mice following IV injection of PC-3M-luc2 dBP4 cells. Kaplan-Meier plot showing percent survival (time to sacrifice) in mice with liver metastases following IV injection of PC-3M-luc2 dBP4 cells. Controls fed normal chow (n=5) or dBP4-expressing mice fed doxycycline chow (200 mg/kg, n=5). Time to sacrifice was based on signs of illness including depressed activity, unkept haircoat, hunched posture or decreased food/water intake (p = ns).
Figure 5.27: Non-fasting blood glucose levels were unchanged post-induction of dBp4. Blood glucose levels were measured in controls fed normal chow (n=5) or dBp4-expressing mice fed doxycycline chow (200 mg/kg, n=5) using AlphaTRAK glucose meter. Pre-treatment and post-treatment blood glucose was measured from the lateral tail vein and upon sacrifice of mice by cardiac puncture, respectively. Data (n=5 mice/group) are expressed as mean ± SEM. A paired t-test was carried out to test for differences between pre-treatment glucose and post-treatment glucose levels for each group (p = ns).
Figure 5.28: Serum IGF I levels were unaffected following dBP4 expression compared with controls in liver metastases formed after IV injection of PC-3M-luc2 dBP4 cells. Serum was collected from NSG mice with PC-3M-luc2 dBP4 liver metastases (IV injection) treated with doxycycline chow (200 mg/kg, n=5) or controls received normal chow (n=5). IGF I levels were measured in duplicate by ELISA in serum Data (n=3 mice/group) are expressed as mean ± SEM. No significance difference in serum IGF I was seen between controls or dBP4-expressing mice Data was analysed by student t-test (p = ns).
5.4 Discussion

dBP4 may prove to be superior to other existing strategies to block IGF signalling in cancer as current therapies (mAbs and TKIs) lead to undesirable side effects such as hyperinsulinaemia and hyperglycaemia due to effects on the IR (Mulvihill et al., 2009). Blocking the IGF I ligand with dBP4 instead of targeting the IGF IR may avoid these side effects.

A Tet inducible system was used to introduce a doxycycline-inducible dBP4 construct into PC-3M-luc2 in order to assess dBP4 in vivo. HEK 293 and PC-3M-luc2 cells were transduced with the pTet-On Advanced plasmid which produces the rtTA protein that switches on dBP4 expression in the presence of doxycycline. pTet-On HEK 293 cells were transfected with a luciferase reporter gene (pLVX Tight Puro-luc) and bioluminescence was only seen in HEK 293 cells upon addition of d-luciferin and doxycycline demonstrating doxycycline inducible expression of luciferase. Western blot analysis showed that the rtTA protein was expressed by PC-3M-luc2 cells transduced with the pTet-On Advanced vector. Following selection of stable clones expressing rtTA, pTet-On PC-3M-luc2 cells were transduced with the pLVX Tight Puro dBP4 response plasmid creating PC-3M-luc2 dBP4 cells. Conditioned medium and cell lysates from doxycycline treated PC-3M-luc2 dBP4 cells showed expression of dBP4 detected using anti-His-tag antibody which differentiates between dBP4 and endogenous IGFBP 4. In PC-3M-luc2 dBP4 conditioned medium, dBP4 was at a lower molecular weight than the cell lysates and this was attributed to cleavage of the signal peptide upon secretion of dBP4. dBP4 was not present in PC-3M-luc2 dBP4 cells without doxycycline; therefore dBP4 was under tight regulation and was not 'leaky' in the absence of doxycycline in vitro.

Once PC-3M-luc2 dBP4 cells were shown to express dBP4 following doxycycline induction, in vivo models of prostate cancer were set up to test the effect of vector-expressed dBP4 on tumour growth and metastasis. A preliminary study was carried out to assess doxycycline-induced dBP4 expression in PC-3M-luc2 dBP4 tumours growing subcutaneously in the right flank of NSG mice. The effects of dBP4 on tumour growth were compared with that of a known IGF IR TKI, NVP-AEW541 for 7 days. Both the subcutaneous and orthotopic models were carried out simultaneously. NVP-AEW541 at the dose used (50 mg/kg) did not inhibit tumour growth in the subcutaneous tumour model or activation of the IGF IR thus suggesting NVP-AEW541 was inactive. In vitro, NVP-AEW541 inhibited IGF-induced proliferation of PC-3M-luc2 cells (Figure 4.10). However, NVP-AEW541 inhibited IGF IR activation in the orthotopic model, but did not inhibit prostate tumour growth. NVP-AEW541 has not been previously tested in prostate cancer models. Therefore, the 50 mg/kg dose used in this study was based on previous published data using different model systems. Numerous studies have shown that NVP-AEW541 inhibits...
tumour growth \textit{in vivo}. Neuroblastoma cell lines (HTLA-230 or S-KN-BE2c cells) were implanted subcutaneously in nude mice and treated with NVP-AEW541 (50 mg/kg). NVP-AEW541 inhibited tumour growth, decreased VEGF mRNA and decreased metastasis formation (Tanno \textit{et al.}, 2006). NVP-AEW541 was also assessed \textit{in vitro} in Ewing's sarcoma (10 cell lines), rhabdomyosarcoma (5 cell lines) and osteosarcoma (8 cell lines). Ewing's sarcoma cells were more sensitive to the effects of NVP-AEW541 compared with rhabdomyosarcoma and osteosarcoma, which is in agreement with the high dependency of Ewing's sarcoma on IGF IR signalling (Scotlandi \textit{et al.}, 2005). In an \textit{in vivo} Ewing's sarcoma model, NVP-AEW541 at a dose of 50 mg/kg was only effective in combination with vincristine and a higher dose (100 mg/kg) was required to see a significant reduction in tumour volume (Manara \textit{et al.}, 2007). Therefore, a higher dose of NVP-AEW541 may have been required in this study. From this, a comparison between dBP4 and NVP-AEW541 could not be made.

IGF I levels were assessed in the serum and tumours of control, dBP4 subcutaneous tumours to determine if dBP4 would cause a compensatory production of IGF I due to blockade of the IGF IR and IGF I by dBP4. Two mice from each group were sacrificed (controls or doxycycline treated) at day 3 and day 7. As only 2 mice from each group were assessed no statistics were performed. Tumours from controls (n=2) or dBP4-expressing mice (n=2) were assessed by Western blotting with anti-His-tag antibody to confirm induction of dBP4 in doxycycline treated mice. dBP4 expression was seen after day 3 and remained expressed at day 7 in the doxycycline treated group. A low level of dBP4 was seen in controls. However, dBP4 was strongly expressed in the doxycycline treated tumours. Serum IGF I did not change in controls or dBP4-expressing mice from day 3 to day 7. Tumour IGF I levels increased in the control tumours from day 3 to day 7. No change in tumour IGF I was reported in dBP4-expressing tumours.

To test the effect of dBP4 on prostate tumour growth and metastasis NSG mice were chosen for implantation of PC-3M-luc2 dBP4 cells. Nude mice have been used for many years for xenograft models, since they lack T lymphocytes, but growth of cancer cells in these mice can be slow (Pretlow \textit{et al.}, 1991) and they have shown a 38 % rate of xenotransplantation of prostate cancer cells (Van Weerden \textit{et al.}, 1996). Many different models have been developed from athymic mice. SCID mice are unable to mount an immune response due to a genetic defect in Chromosome 16 responsible for deficient activity of an enzyme involved in DNA repair, which inhibits B and T lymphocyte differentiation, leading to loss of these cell types (Bosma \textit{et al.}, 1983). In order to increase the immunodeficient properties of the SCID model, SCID mice were crossed with NOD mice, which are deficient in natural killer cells, complement and functional antigen presenting cells (Greiner \textit{et al.}, 1995). The resulting NOD-SCID mice were more immunodeficient than SCID.
mice and accepted foreign tissue with a higher success rate. Following injection of human CEM T-lymphoblastoid cells, splenic engraftment of these cells was fourfold greater in NOD-SCID mice than in SCID mice (Shultz et al., 1995). Finally, NSG mice were developed by crossing NOD-SCID mice with IL-2R γ null mice and are the most severely immunodeficient mouse available (Ito et al., 2002). NSG mice have a higher engraftment rate than NOD-SCID mice (13/13 compared with 8/13, when human haematopoietic stem cells were injected) (Shultz et al., 2005). When E0006A cells (prostate cancer cells) were implanted subcutaneously in NSG or nude mice, tumours stopped growing in the nude mice after 6 - 16 weeks, whereas tumours in the NSG mice continued to grow and did not regress (D'antonio et al., 2010). The NOD background should not effect non-fasting blood glucose readings as diabetes formation in these mice is due to an autoimmune response resulting in leukocyte infiltration of pancreatic islets (Tang et al., 2008). Crossing of the NOD mice with SCID mice leads to a severely immunodeficient strain, which do not develop diabetes due to lack of an immune system.

Using a larger cohort of NSG mice the long-term effect of dBP4-expression on subcutaneous PC-3M-luc2 dBP4 tumours was completed. Tumour growth was monitored by caliper measurements and BLI. Tumours were monitored for 20 days after initiation of treatment using BLI, but tumours became visibly necrotic after day 20 and as luciferase is only expressed in viable cells BLI would therefore under estimate tumour burden. In addition, BLI was not used in large subcutaneous tumours because the location results in saturation of signal and non-linear correlation with tumour size. However, inhibition of angiogenesis can also cause necrosis. Transfection of rat C6 glioma cells with anti-sense VEGF cDNA implanted subcutaneously in nude mice led to a decreased number of blood vessels and increased degree of necrosis within tumours relative to control cells (Saleh et al., 1996). Therefore, following day 20 of treatment inhibition of VEGF by dBP4 may have led to necrosis in dBP4-expressing tumours. Caliper measurements were more reliable than BLI after this point. BLI images from each treatment group showed that dBP4-expressing subcutaneous tumours were smaller than controls. This was seen for all mice within the dBP4-expressing cohort. This was also reflected in caliper MTD measurements of dBP4-expressing subcutaneous tumours growing significantly slower than controls. However, when the average bioluminescence was calculated for each group no significant difference in bioluminescence was seen. Even though an increase in bioluminescence was seen in all groups, BLI may not have efficiently measured tumour size as large tumours saturate the signal leading to non-linear correlation with tumour size.

Mice bearing dBP4-expressing tumours survived (37 ± 2.4 days) significantly longer than controls (27.8 ± 2.4 days) with an increase of 33 %. Survival was based on time to reach 20 mm MTD or when signs of impaired mobility were reported. In a previous study
using the 4T1.2 breast cancer model where 4T1.2 cells were transiently transfected with dBP4 and injected into the mammary fat pad, tumours expressing dBP4 grew significantly slower than controls or cells expressing wildtype IGFBP 4 (Ryan et al., 2009). Damon et al (1998) implanted androgen receptor negative M12 prostate cancer cells transfected with wildtype IGFBP 4 subcutaneously in nude mice. A delay in tumour onset was seen initially relative to controls. However, by week 7 one of the IGFBP 4 expressing clones did not show a significant difference in tumour formation relative to controls. This suggests that IGFBP 4 may be cleaved by proteases such as PAPP-A which is highly expressed in the skin (Chen et al., 2003). Therefore, a protease resistant IGFBP 4 may have been more useful. Also the wildtype IGFBP 4 was expressed in the M12 cells from the beginning which does not reflect the clinical situation as individuals are not given treatment before a tumour is established. Therefore, our study better reflects the clinical situation where tumours have become established and treatment is initiated accordingly. In the subcutaneous prostate cancer model, survival was based on time to sacrifice as few tumours were allowed to reach 20 mm diameter due to discomfort and leg paralysis. A difference in tumour growth was seen from treatment day 14 - day 20.

Hyperglycaemia leads to excessive glucose circulating in the blood and is most commonly associated with diabetes (Rossetti et al., 1993). Low insulin or insulin resistance results in an inability to regulate and maintain blood glucose levels within normal range. Certain drugs such as anti-psychotics induce hyperglycaemia (Goldstein et al., 1999), in particular a number of mAbs and TKIs to the IGF IR induce mild-moderate hyperglycaemia (Higano et al., 2007, Smith et al., 2008) due to cross-reactivity with the IR as it shares 60 % homology with the IGF IR (Ullrich et al., 1986). PC-3M-luc2 cells formed heterodimers with the IR and upon stimulation with IGF (E3R) an increase in phosphorylated IR was seen (Figure 3.10). However, hybrid receptors containing IGF IR and IR are thought to act as an IGF IR (Johansson and Arnqvist, 2006) and have a higher affinity for IGF I than insulin, hybrid receptors bind 20 times more IGF I than insulin (Soos et al., 1993). This suggests that blocking the IGF I ligand binding to these hybrid receptors should not effect metabolic functions. In this study, as hyperglycaemia is a common side effect of IGF IR blockade the effect of dBP4 on non-fasting blood glucose was measured in all mice before initiation of treatment and before sacrifice. Blood glucose was measured from non-fasting mice as blood glucose >200 mg/dL (11 mmol/L) is indicative of hyperglycaemia in non-fasting mice. Measurement of non-fasting glucose was used previously to genetically dissect diabetes-related traits, hyperglycaemia was seen as a non-fasting glucose reading of >200 mg/dL (11 mmol/L) (Kobayashi et al., 2003). Non-fasting glucose levels in NSG mice bearing dBP4-expressing subcutaneous tumours were significantly increased post-induction of dBP4 relative to pre-treatment glucose levels. Blood glucose of dBP4-expressing mice did not
exceed 11 mmol/L suggesting that mice were not hyperglycaemic. When serum IGF I levels were measured in the same mice, a significant increase in serum IGF I was seen in NSG mice bearing dBP4-expressing tumours. Along with insulin, IGF I is known to regulate glucose levels within the body. IGF I increases insulin sensitivity thus decreasing glucose levels (Zenobi et al., 1992). From this it would suggest that increased IGF I levels should decrease glucose levels. As mentioned, serum IGF I levels were increased in mice bearing dBP4-expressing tumours relative to controls. Contrary to this, IGF I levels in dBP4-expressing tumours were significantly reduced relative to control tumours. Therefore, tumour IGF I levels did not differ over time within groups as shown in the preliminary study but differences were seen between groups. As tumour IGF I levels were significantly decreased, it suggests that dBP4 may affect IGF I levels within the tumours. Also, it may be possible that IGF I bound to dBP4 was not detected in the ELISA assay. dBP4 may decrease tumour IGF I due to an increased reservoir of local IGF I; therefore tumours may reduce expression of IGF I. A decrease in tumour IGF I may lead to a compensatory mechanism to overcome reduced local IGF I levels. This mechanism may involve an increase in GH secretion, which raises IGF I-hepatic production leading to elevated serum IGF I. High levels of GH can lead to insulin resistance resulting in hyperglycemia (Takala et al., 1999). This may explain why an increase in serum IGF I and blood glucose was seen in mice bearing dBP4-expressing subcutaneous tumours.

At the molecular level, IGF I signals via the IGF IR activating Akt and ERK. In the subcutaneous tumour mouse model, dBP4-expressing tumours showed a decrease in activated IGF IR relative to controls. Phospho Akt was decreased in the dBP4-expressing tumours relative to controls. Phospho ERK was not inhibited by dBP4.

Angiogenesis is important in tumour growth as blood vessels supply the tumour with oxygen and nutrients (Folkman et al., 1971). VEGF-A is the main factor involved in angiogenesis. There are several isoforms expressed within the body, VEGF_{121}, VEGF_{145}, VEGF_{165}, VEGF_{189}, VEGF_{206} and a splice variant of VEGF_{165} called VEGF_{165b} which has anti-angiogenic properties (Woolard et al., 2004) compared with the other isoforms. VEGF_{121} is acidic and does not bind heparin making it a diffusible protein (Houck et al., 1992). VEGF_{165} is the most abundant VEGF isoform and is the most potent in stimulating angiogenesis. It is a secreted protein, but can also bind the cell surface and extracellular matrix (Park et al., 1993). VEGF_{189} and VEGF_{206} are basic and bind heparin which results in these proteins becoming sequestered in the extracellular matrix (Houck et al., 1992). IGF I stimulates production of VEGF (Poulaki et al., 2003, Slomiany and Rosenzweig, 2004). Therefore, inhibition of IGF I by dBP4 may inhibit VEGF production. In the subcutaneous tumours, VEGF_{165} and VEGF_{189} were the main isoforms reported as the antibody only detects VEGF_{121}, VEGF_{165} and VEGF_{189}. The control tumours only expressed VEGF_{165}. 182
whereas VEGF<sub>165</sub> expression was abolished in dBP4-expressing tumours suggesting that dBP4 inhibited IGF I induced production of VEGF. The VEGF produced by the tumours may have been predominantly produced by the PC-3M-luc2 dBP4 cells, but the murine host cells may have also contributed to the VEGF production. The murine isoforms consist of VEGF<sub>120</sub> (14 kDa), VEGF<sub>164</sub> (23 kDa) and VEGF<sub>188</sub> (27 kDa) (Ng et al., 2001). The difference in length also leads to a difference in size. However, it is difficult to distinguish between the human and murine isoforms as the murine isoforms are only one amino acid shorter. Previously saw that dBP4 decreased angiogenesis in vitro (Chapter 4) and in vivo (Ryan et al., 2009).

Subcutaneous tumours were tested for expression of dBP4 following doxycycline treatment so as to determine if tumours maintained dBP4 expression at the end of the study. Three out of the 4 tumours tested showed dBP4 expression. The lack of inhibition seen in pERK by dBP4 was not due to loss of dBP4 expression. As previously mentioned inhibition of the IGF IR may lead to cross talk from alternative pathways. As discussed in section 3.3, the IGF IR forms heterodimers with the IR and EGFR. Activation of Akt and ERK may be facilitated by other growth factor signalling via other receptors (Nahta et al., 2005, Riedemann et al., 2007).

The second model to test dBP4 in vivo was an orthotopic model, implantation of PC-3M-luc2 dBP4 cells into the prostate. This model reflects human prostate cancer and reflects the behaviour of human prostate cancer. Similar to the subcutaneous model, BLI images showed that dBP4-expressing prostate tumours grew significantly slower than controls by day 19 of treatment. This was seen as a decrease in BLI, which is also indicative of a decrease in cell viability as viable cells will only bioluminescence (Rehemtulla et al., 2000). Prostate tumours were smaller than subcutaneous tumours so necrosis was probably not a factor in the decreased BLI and in this model BLI probably reflects tumour size. dBP4 expression in prostate tumours did not increase survival of mice relative to controls. Survival was based on the point where mice showed signs of depressed activity, unkept haircoat, hunched posture or decreased food/water intake. No difference in survival was seen as prostate tumours impinge on other organs in the abdomen such as the bladder and intestine, therefore mice became sick sooner compared with those implanted with subcutaneous PC-3M-luc2 dBP4 cells.

Non-fasting glucose levels were measured in prostate tumour bearing mice pre and post-treatment induction. Control mice showed a significant decrease in non-fasting glucose upon sacrifice whereas no change in glucose levels were seen in dBP4-expressing mice post-treatment relative to pre-treatment glucose levels. A decrease in blood glucose in controls from the orthotopic model may be attributed to control mice not eating as a result of illness, but hypoglycaemia is also associated with cancer as tumours utilise the majority of available glucose putting a strain on the host's ability to maintain normal glucose levels.
Hypoglycaemia may indicate large tumour burden in the control mice compared with dBP4-expressing mice, which was seen in patients with advanced gastrointestinal stromal tumours (Pink et al., 2005). This is supported by the BLI where controls showed a significant increase in bioluminescence relative to dBP4-expressing tumours. When assessing IGF I levels, in contrast to that seen in the subcutaneous model, serum IGF I levels did not change in dBP4-expressing mice relative to controls, but a significant decrease in tumour IGF I was observed in dBP4-expressing tumours relative to controls which correlates with that seen in the subcutaneous model. Similar to the subcutaneous model the decrease in tumour IGF I may have been attributed to the IGF I ELISA not detecting IGF I bound to dBP4.

To determine the effect of dBP4 at a cellular level, activated IGF IR, Akt and ERK was assessed in prostate tumours. Activated IGF IR was decreased in dBP4-expressing tumours and NVP-AEW541-treated tumours. However, no inhibition of phospho Akt (pAkt) and ERK (pERK) was seen in dBP4-expressing tumours. A higher level of pAkt was noted in dBP4-expressing tumours. However, unequal loading (α-tubulin) shows higher protein levels in dBP4-expressing prostate tumours. dBP4 remained expressed at the end of the study; therefore lack of pAkt and pERK inhibition was not due to loss of dBP4 expression within tumours.

To further examine the effect of dBP4 in vivo two models of experimental metastasis were used which give rise to bone, lung and liver metastases. Bone metastasis is most commonly associated with prostate cancer (Dai et al., 2008). Each model was used to give a greater chance of metastases formation in mice. Unfortunately, few mice developed bone metastases with the majority of metastases formed in the liver. PC-3M cells were derived from a mouse liver metastasis following injection of parental PC-3 cells into mouse spleen (Kozlowski et al., 1984). Therefore, PC-3M may have had a higher propensity to form metastasis in the liver. However, PC-3M cells were injected into nude mice via intracardiac injection in order to monitor tumour relapse and metastasis, bone metastasis formation was seen in these mice (Jenkins et al., 2003).

Following intracardiac injection of PC-3M-luc2 dBP4, only 6 developed metastases, 3 controls and 3 dBP4-expressing. Mice were monitored closely using BLI, dBP4-expressing liver metastases was localised to one area within in the liver 8 days post dBP4 induction whereas one of controls showed the liver metastases spread throughout the liver by day 8. Survival was based on the point where mice showed signs of depressed activity, unkept haircoat, hunched posture or decreased food/water intake. Mice with dBP4-expressing liver metastases were sacrificed sooner than control mice, this may be due to dBP4-expressing mice having higher BLI at day 0, but no significant difference in the Kaplan Meier curve was seen. This may have been due to small n numbers. Non-fasting glucose levels did not
change in controls or dBP4-expressing liver metastases mice post dBP4 induction relative to pre-treatment glucose levels. There was no difference in serum IGF I levels in mice bearing dBP4-expressing liver metastases relative to control mice. Therefore dBP4 was not affecting systemic IGF I.

Following IV injection of PC-3M-luc2 dBP4, 10 mice developed liver metastases with bone metastases present in some of the mice. However, the BLI for the bone metastases appeared to fluctuate between imaging days due to the position of the mice within the chamber, therefore they were not included in the study. Metastases formation and growth post dBP4 induction was monitored by BLI up to day 14. dBP4-expressing liver metastases appeared localised to one area within the liver post dBP4 induction whereas the controls showed an increase in metastatic burden. Survival was based on the point where mice showed signs of depressed activity, unkept haircoat, hunched posture or decreased food/water intake. dBP4 did not have a significant effect on survival. However, n numbers may have been too small to see an effect. Non-fasting glucose levels did not change in controls or dBP4-expressing liver metastases mice post dBP4 induction relative to pre-treatment glucose levels. There was no difference in serum IGF I levels in mice bearing dBP4-expressing liver metastases relative to control mice. Therefore, dBP4 was not affecting systemic IGF I. Both the intracardiac and IV experimental metastasis models showed similar patterns of tumour growth. However, the effect of dBP4 on metastasis growth could not be deduced from either of these models as tumour burden was large before induction of dBP4, therefore long-term effect of dBP4 could not be fully completed as mice were sacrificed early in the study. Also, the liver is the main source of IGF I within the body (Sjögren et al., 1999), therefore the PC-3M-luc2 dBP4 cells may not have produced enough dBP4 to block IGF I effects. As the PC-3M cells were originally derived from a liver metastasis (Kozłowski et al., 1984) this may also explain the high level of liver metastasis seen in the intracardiac and IV experimental metastasis models.

The key findings from this chapter were that dBP4 slowed tumour growth in the subcutaneous and prostate tumour models. dBP4 increased survival in the subcutaneous model. IGF I levels were decreased in tumours from both the subcutaneous and prostate tumour models. At the cellular level, dBP4 decreased activation of pIGFR and phospho Akt in the subcutaneous model. dBP4 inhibited VEGF production in subcutaneous tumours. However, dBP4 only decreased pIGFR in the orthotopic model. NVP-AEW541 inhibited IGF IR activation, but had no effect on prostate tumour growth. As IGF I is involved in angiogenesis and in Chapter 4 dBP4 inhibited endothelial cell tubule formation, it would be of benefit to assess dBP4 effects on tumour angiogenesis. dBP4 was shown to inhibit 4T1.2 breast cancer cell growth in vivo, overexpression of dBP4 in mice showed a reduction in tumour growth and increased survival relative to controls as well as anti-angiogenic effects.
(Ryan et al., 2009). A comparison between dBP4 and NVP-AEW541 could not be made as NVP-AEW541 was either inactive in the subcutaneous model or a higher dose was required in order to see an inhibition in tumour growth in both the subcutaneous and orthotopic models. However, this may suggest that blocking IGF I ligand is more effective than inhibition of the tyrosine kinase domain in PC-3M-luc2 cells. Unfortunately, the experimental metastasis models did not give insight into the effects of dBP4 in metastasis. Therefore, this study would need to be repeated and dBP4 induction carried out sooner to determine dBP4 effect on inhibiting metastasis formation. The data presented shows that dBP4 inhibits IGF I, thus inhibiting tumour growth suggesting that dBP4 may have a therapeutic value in prostate cancer.
Chapter 6
Concluding Discussion
One of the major clinical challenges in treating prostate cancer is the development of androgen independent disease and metastasis. Therapies used against the primary tumour are often less effective against Al disease and metastasis due to changes in gene expression as tumours progress to an androgen independent state (Best et al., 2005). Ten androgen independent tumours and 10 androgen dependent tumours were assessed for differences in gene expression. Two main genes were found to be different between androgen independent and androgen dependent tumours: macromolecule biosynthesis was down-regulated and cell adhesion was up-regulated in androgen independent tumours relative to androgen dependent tumours. Other differentially expressed genes linked to angiogenesis, cell adhesion, apoptosis, oxidative stress and hormone response were identified along with 9 regions of potential chromosomal deletion in androgen independent tumours (Best et al., 2005). Androgen independent prostate cancer typically develops 18 - 24 months after hormone ablation therapy (Rau et al., 2005). Once testosterone production is blocked tumours adapt by numerous pathways to overcome the growth inhibition (Mc Donnell et al., 1992, Miyamoto et al., 1998, Yeh et al., 1999). This leads to a more aggressive and invasive phenotype, ultimately leading to bone metastasis (Gingrich et al., 1997). Interactions between the tumour cells and the host microenvironment are crucial in the establishment of tumours and metastases (Fidler et al., 2007). Therefore, targeting factors involved in this interaction may be key in inhibiting tumour growth and metastasis (Allavena et al., 2005, Bäuerle et al., 2008, Espinoza et al., 2011). One potential therapeutic target is the IGF signalling pathway, which is involved in differentiation, transformation, proliferation and angiogenesis (Kaleko et al., 1990, Moser et al., 2008, Stewart et al., 1990, Valentinis et al., 1999). The IGF pathway has been implicated in tumour growth and metastasis (Parker et al., 2002, Tang et al., 2003). In this study, plasma IGF I levels were assessed in BPH, Gleason score 5 and Gleason score 7 prostate cancer patients. Although no significant difference in plasma IGF I was identified between BPH and prostate cancer patients this may be due to small patient numbers used compared with other studies of IGF I in prostate cancer as this study had a total of 33 patient samples compared with patient numbers of n=520, n=447, n= 210, respectively (Chan et al., 1998, Stattin et al., 2000, Wolk et al., 1998). Those studies have shown a positive association between elevated IGF I levels and prostate cancer risk (Chan et al., 1998, Stattin et al., 2000, Wolk et al., 1998). In those studies healthy individuals were used as controls whereas in this study BPH patients were used. However, it has been reported that there is no association between IGF I levels and BPH suggesting that IGF I does not play a role in the development of BPH (Mantzoros et al., 1997). As PAPP-A cleaves IGFBP 4 releasing IGF I (Lawrence et al., 1999), plasma PAPP-A was also measured in the same patient samples (n=33). PAPP-A proteolytic activity in vitro accelerated anchorage independent growth and
increased invasion through Matrigel of PAPP-A overexpressing SKOV3 (ovarian cancer) cells relative to SKOV3 cells expressing a mutant PAPP-A with no proteolytic activity (Boldt and Conover, 2011). This suggests that PAPP-A may be important in tumour growth and invasion as a consequence of proteolysis of IGFBP 4 leading to increased IGF I activity. In this study, PAPP-A was significantly higher in Gleason score 5 patients relative to BPH patients. PAPP-A levels have not been previously assessed in prostate cancer patients. However, no association between IGF I levels and PAPP-A levels was seen. PAPP-A indirectly reflects IGF I released from IGFBP 4 whereas the ELISA used in this study measured total IGF I and over 70 % of circulating IGF I is bound to IGFBP 3 in a complex with ALS (Baxter et al., 1989). PAPP-A is associated with acute coronary syndromes and although elevated PAPP-A was reported in patients with unstable angina and acute myocardial infarction, no association was seen between PAPP-A and total IGF I levels, whereas a weak positive association was seen between free IGF I and PAPP-A (Bayes-Genis et al., 2001). Therefore, measurement of free IGF I in prostate cancer patients may also identify an association between plasma PAPP-A and plasma IGF I levels. PAPP-A was elevated in the serum of lung cancer patients (75 men and 8 women) and a significant increase was seen relative to healthy controls (30 men and 3 women). In the same study a positive association was reported between PAPP-A levels and age (Bulut et al., 2009) whereas in this study a negative association was seen between PAPP-A and age. Therefore, PAPP-A measurement may be a useful prognostic marker for younger men with early stage prostate cancer. However, a study using healthy controls and a higher patient number will be necessary to validate that PAPP-A levels are elevated in prostate cancer patients.

The IGF IR has also been implicated in prostate cancer. Increased IGF IR expression has been shown in prostate cancer tissue relative to benign prostatic epithelium and remains expressed in metastatic disease (Hellawell et al., 2002). An association between IGF IR expression and Gleason grade was identified in this study by staining TMAs of prostate cancer tissue (45 tumours) with anti-IGF IR antibody. A significant increase in IGF IR staining was seen in prostate cancer tissue compared with BPH/normal tissue. IGF IR staining was highest in Gleason grade 3, but decreased in Gleason grade 5 as previously reported by Liao et al (2005) (56 tumours). This further supports the involvement of the IGF pathway in prostate cancer. A decrease in IGF IR expression has been demonstrated in aggressive tumours, which is associated with impaired AR activity (Schayek et al., 2010a). Enhanced AR binding to the IGF IR promoter was reported in wildtype AR-overexpressing M12 prostate cancer cells relative to mutant AR-expressing M12 prostate cancer cells. In a xenograft model, total and phosphorylated IGF IR levels progressively decreased as prostate cancer cells became more tumourigenic and metastatic (Schayek et al., 2010b).
Therefore, immunohistochemical analysis of the IGF IR may be of value in pre-therapeutic assessment of prostate cancer aggressiveness and could contribute to an improved initial patient management.

Due to accumulating evidence supporting a role for the IGF pathway in cancer initiation and progression (De Graff et al., 2009, Kaleko et al., 1990, Kanety et al., 1993, Kim et al., 2007, Parker et al., 2002, Peiro et al., 2011, Rochester et al., 2004) attention has turned towards developing strategies to inhibit the IGF pathway. The 2 main strategies that are furthest along in clinical development are mAbs and TKIs targeting the IGF IR (Higano et al., 2007, Lacy et al., 2008, Lindsay et al., 2009, Smith et al., 2008). In clinical trials they have proven to be well tolerated. IGF antagonists such as anti-IGF IR mAbs, which act by blocking IGF binding to the IGF IR may also alter insulin action at the IR leading to development of hyperglycaemia (Haluska et al., 2007). Targeting the IGF I ligand should avoid cross reactivity with the IR. The IGFBPs are specific for the IGFs and should not affect insulin signalling. As the IGFBPs bind IGFs with higher affinity (kd=10^-10-10^-11 M) than IGF IR (kd=10^-8-10^-9 M) (Kiefer et al., 1992, Oh et al., 1993b), they should effectively compete with the receptor for IGF ligand binding.

The strategy used in this study involved the use of a protease resistant form of IGFBP 4 (dBP4) to inhibit IGF I effects. IGFBP 4 is solely inhibitory to IGFs unlike other IGFBPs, which have IGF dependent and independent effects that can either inhibit or enhance IGF I function depending on the context. IGFBP 3 induced apoptosis via a caspase-mediated pathway independent of IGF I in MCF-7 cells transfected with a mutant IGFBP 3 that could not bind IGF I (Kim et al., 2004). Human ectocervical cells, immortalised by retroviral transduction with the human papillomavirus type 16 E6/E7 oncogenes expressed higher levels of IGFBP 3 mRNA relative to parental cells. Late-passage, immortalized E6/E7-transduced cells secreted high levels of IGFBP 3 and exhibited an increased sensitivity to IGF I, including increased phosphorylation of the IGF IR, increased DNA synthesis and cell proliferation (Baege et al., 2004). The majority of circulating IGF I is bound to IGFBP 3 in a complex with ALS which cannot cross endothelial barriers thus impairing delivery of recombinant IGFBP 3 to tumours (Payet et al., 2004). There are a number of reasons why a protease resistant IGFBP 4 may be a superior way of blocking IGF signalling than other recombinant IGFBPs or mAbs directed against the IGF IR. Firstly, IGFBP 4 is small enough (28 - 34 kDa) to cross endothelial barriers (Boes et al., 1992) unlike IGFBP 3 which is bound as a large complex with an acid labile subunit (Payet et al., 2004) and should therefore penetrate tumour tissue more effectively. Secondly, mAbs directed against the IGF IR can interfere with insulin signalling due to binding to heterodimers of IGF IR and IR (Pandini et al., 2007, Treadway et al., 1991). Thirdly, as dBP4 is resistant to PAPP-A it may have a relatively long half-life in vivo (Eldridge et al.,
2009). One of the first uses of a protease resistant IGFBP 4 was in smooth muscle cells of transgenic mice. Expression of protease resistant IGFBP 4 transgene under the control of the smooth muscle actin promoter led to muscle hypotrophy and decreased aortic, bladder and stomach weight relative to mice expressing the wild type IGFBP 4 transgene (Zhang et al., 2002a). A protease resistant rat IGFBP 4 was previously assessed by the Harmey group in the mouse 4T1.2 breast cancer model. Mammary tumours expressing rat dBP4 grew significantly slower than controls or tumours expressing wildtype rat IGFBP 4 along with increased survival. Inhibition of tumour growth was accompanied by increased endothelial cell apoptosis in dBP4-expressing tumours (Ryan et al., 2009). As IGF I is implicated in prostate cancer growth and metastasis we evaluated the effects of dBP4 expression in prostate cancer models.

PC-3M-luc2 cells were chosen for in vitro and in vivo work. These cells are androgen independent (Tilley et al., 1990) which reflects advanced stage prostate cancer. The PC-3M-luc2 cells expressed the IGF IR and IGF (E3R) increased their proliferation. Inhibition of the IGF IR in vitro with TKIs AG1024 and NVP-AEW541 led to a significant decrease in baseline proliferation and IGF-induced proliferation, which suggested that inhibition of IGF signalling with dBP4 may be effective in PC-3M-luc2 tumour models in vivo. IGF I was shown to increase metabolic activity in the MTS assay whereas no effect was seen in the BrdU proliferation assay. This difference in effect may be due to a difference in seeding density. Furthermore, MTS assay measures metabolic activity assuming a direct correlation with cell number and IGF I may increase metabolic activity as well as DNA synthesis which is measured by the BrdU assay.

In the Ryan et al (2009) study, rat dBP4 was expressed from a plasmid vector under control of a constitutive promoter. To better reflect the clinical situation where treatment can only be initiated after detection of a tumour, in this study dBP4 expression was induced once PC-3M-luc2 dBP4 tumours were established. Furthermore, in this study dBP4 was introduced using a lentiviral vector to integrate dBP4 into the genome for sustained IGFBP 4 expression. An inducible Tet-On system was used in the PC-3M-luc2 cells where dBP4 expression was induced by doxycycline. The effectiveness of dBP4 in inhibiting tumour growth was compared with a well established TKI, NVP-AEW541. Tanno et al (2006) showed that NVP-AEW541 inhibited neuroblastoma cell growth in nude mice and decreased tumour microvascularisation relative to controls. Quantitative real-time PCR detected a significant down-regulation of VEGF mRNA in response to NVP-AEW541 relative to controls (Tanno et al., 2006). The effect of dBP4 on tumour growth was monitored in all models by IVIS bioluminescence imaging as well as caliper measurements in the subcutaneous model. A number of studies have used the IVIS system to assess drug effects in vivo (Zeng et al., 2010) and to monitor tumour relapse following drug withdrawal (Jenkins et al., 2003).
This system allowed individual mice to be monitored and compared throughout the study. NSG mice were chosen for the in vivo studies as they are more immunodeficient than nude, NOD and SCID mice (Ito et al., 2002) and have a high engraftment rate compared with other xenograft models (Shultz et al., 2005). The MTD of PC-3M-luc2 dBP4-expressing subcutaneous tumours was significantly smaller than controls. Therefore, dBP4-expressing tumours grew significantly more slowly than controls. A similar effect was seen when PC-3M-luc2 dBP4 cells were implanted into the prostate. A significant decrease in bioluminescence was observed in dBP4-expressing tumours relative to controls. Both of these results are consistent with the mouse 4T1.2 breast cancer model, which showed dBP4-expressing tumours were significantly smaller than controls or wildtype IGFBP 4-expressing tumours (Ryan et al., 2009). Contrary to this, treatment of PC-3M-luc2 cells in vitro with dBP4 did not inhibit proliferation as IGF I did not stimulate proliferation of PC-3M-luc2 cells in this experiment such that it was technically impossible to show the effect of dBP4 on IGF-induced proliferation in vitro. However, dBP4 inhibited endothelial cell proliferation (Ryan et al., 2009) and inhibited IGF-induced tubule formation. dBP4 did not increase survival in the prostate tumour bearing mice whereas dBP4 enhanced survival by 33 % in subcutaneous tumour bearing mice relative to controls. This suggests that the anatomical site of the tumour may have been important in determining survival as prostate tumours impinge on other organs within the abdominal cavity whereas subcutaneous tumours are located externally on the flank and do not interfere with other organs. dBP4-expressing subcutaneous and prostate tumours expressed 0.6 ng/mg and 0.5 ng/mg total IGF I, respectively. In addition, serum IGF I in dBP4-expressing prostate tumour bearing mice was lower than dBP4-expressing subcutaneous tumour bearing mice (690 ng/ml vs. 808 ng/ml, respectively). Therefore, the failure of dBP4 to increase survival within the prostate tumour group is not related to IGF I levels.

NVP-AEW541 did not inhibit tumour growth in either of the prostate cancer models but it did inhibit activation of the IGF IR in the orthotopic model. In vitro, NVP-AEW541 inhibited IGF-induced proliferation of PC-3M-luc2 cells. Other studies have used the same dose of NVP-AEW541 used in this study. Tanno et al (2006) used a dose of 50 mg/kg NVP-AEW541 in a subcutaneous model of neuroblastoma (HTLA-230 or S-KN-BE2c cells). NVP-AEW541 inhibited tumour growth, decreased VEGF mRNA and decreased metastasis formation. Similarly, Moser et al (2008) showed that NVP-AEW541 (50 mg/kg) inhibited growth of BxPC-3 pancreatic cancer cells in a subcutaneous and orthotopic model of pancreatic cancer. Conversely, in an in vivo Ewing’s sarcoma model, NVP-AEW541 at a dose of 50 mg/kg was ineffective and a higher dose (100 mg/kg) was required to see a significant reduction in tumour volume (Manara et al., 2007). Therefore, a higher dose of
NVP-AEW541 may have been required to inhibit tumour growth in both prostate cancer models. Therefore, no conclusions could be drawn from the NVP-AEW541 groups.

As hyperglycaemia is a common side effect associated with therapies blocking the IGF IR (Haluska et al., 2007) non-fasting blood glucose levels were measured. Non-fasting glucose levels >200 mg/dL (11 mmol/L) are indicative of hyperglycaemia in non-fasting mice and fasting causes a decrease in IGF I levels (Lee et al., 2010), which could have interfered with the study. The IGF IR formed hybrid receptors with the IR in the PC-3M-luc2 cells in vitro. Following stimulation with IGF (E3R), an increase in phosphorylated IR was observed in PC-3M-luc2 cells. Due to this interaction, it was important to measure blood glucose levels following dBP4 induction. Interestingly, blood glucose levels were elevated post-induction of dBP4 in mice bearing subcutaneous tumours relative to pre dBP4 induction. However, the increased glucose levels did not reach hyperglycaemic levels (>11 mmol/L) (Kobayashi et al., 2003). The involvement of IGFBP 4 in glucose homeostasis has not been established. IGFBP 1 is the main binding protein involved in glucose homeostasis. Impaired glucose homeostasis was reported in transgenic mice that overexpressed IGFBP 1. Overexpression of IGFBP 1 led to hyperglycaemia, which was attributed to inhibition of IGF I (Rajkumar et al., 1996). However, no change in pre or post-treatment non-fasting glucose was seen in mice bearing dBP4-expressing prostate tumours or in the experimental metastasis models. Moreover, control mice bearing prostate tumours showed a significant decrease in blood glucose relative to pre-treatment levels. The absence of hypoglycaemia in the mice bearing dBP4-expressing prostate tumours might indicate less tumour burden than controls. However, as dBP4 was expressed locally within the tumours and metastases it may not have reached systemic circulation. Systemic administration of dBP4 should give more insight into whether dBP4 effects blood glucose as other therapies (mAbs and TKIs) where hyperglycaemia has been reported are given systemically (Haluska et al., 2007, Lindsay et al., 2009). Future studies should include assessing IR activation in the tumours. From this data, the control prostate tumour bearing mice were suffering from hypoglycaemia (<5 mmol/L) due to an increased tumour burden (Pink et al., 2005). In 142 consecutive patients with hepatocellular carcinomas, 38 were found to have hypoglycaemia. In 124 of the 142 patients, the tumour was rapidly growing and poorly differentiated and there was rapid wasting and profound muscle weakness. Hypoglycaemia developed in 17% within two weeks of death and was associated with a slow decrease in blood glucose on fasting. Hypoglycaemia was a consequence of a progressive increase in demand for glucose by the tumour (Mcfadzean and Yeung, 1969). Hypoglycaemia induced by non-pancreatic tumours is termed non-islet cell tumour hypoglycaemia, which prostate tumours have been categorised as (Marks and Teale, 1998). Therefore, the subcutaneous tumours may not have resulted in hypoglycaemia as tumour burden increased due to site of implantation.
Serum IGF I levels and tumour IGF I levels were assessed to determine if dBP4 affected IGF I systemically and locally. Tumour IGF I levels were significantly decreased in dBP4-expressing subcutaneous and prostate tumours. Serum IGF I levels were elevated in mice bearing subcutaneous dBP4-expressing tumours relative to controls, whereas mice bearing prostate dBP4-expressing tumours or liver metastases showed no change in serum IGF I levels relative to controls. These data suggest that dBP4 reduced local, but not systemic IGF I levels, thus inhibiting tumour growth, which was reflected in the subcutaneous MTD and prostate tumour BLI.

At a cellular level, dBP4 decreased activated IGF IR and decreased phospho Akt in the subcutaneous and only decreased pIGFR in the orthotopic PC-3M-luc2 dBP4 tumours. In vitro, PC-3M-luc2 cells treated with IGF I or IGF (E3R) showed an increase in phospho Akt which was inhibited by wortmannin. Therefore, blockade of IGF I by dBP4 would be expected to inhibit Akt activation in PC-3M-luc2 cells. PC-3M-luc2 cells are PTEN negative (Vliestra et al., 1998) therefore the Akt pathway may be predominant over the MAPK pathway in these cells. This was reflected in vitro where IGF I or IGF (E3R) did not increase activation of ERK in PC-3M-luc2 cells. Therefore, blockade of IGF I by dBP4 was not expected to inhibit ERK activation.

VEGF is a potent factor involved in angiogenesis. VEGF expression is induced by hypoxia via activation of HIF 1α (Forsythe et al., 1996) or by growth factors such as IGF I (Warren et al., 1996, Poulaki et al., 2003). Due to its involvement in tumour growth and metastasis, therapies against VEGF have been developed. In prostate cancer, VEGF expression has been positively associated with Gleason score (Mazzucchelli et al., 2000). Overexpression of VEGF in androgen independent PC-3 cells under hypoxic conditions showed increased invasion through Matrigel in the presence of HUVECs relative to wildtype PC-3 cells (Ackerstaff et al., 2004). VEGF has also been shown to promote osteoblast activity. C4-2B prostate cancer cells which expressed VEGF165 and VEGF169 were injected into the tibia of mice and after the tumours grew for 6 weeks, mice were administered the VEGFR inhibitor, PTK787. PTK787 decreased intratibial tumour burden and C4-2B-induced osteoblastic activity (Kitagawa et al., 2005). The androgen independent prostate cancer cell line DU145 was implanted subcutaneously in SCID mice and following treatment with a monoclonal antibody to VEGF a significant decrease in tumour growth and metastasis was seen relative to controls (Melnyk et al., 1999). In this study, it was shown in vitro that IGF (E3R) increased VEGF165 production by PC-3M-luc2 cells relative to untreated controls. In human endothelial cells, dBP4 significantly decreased IGF-induced tubule formation (angiogenesis) relative to untreated controls, or IGF I treated endothelial cells. This supports a role for dBP4 in the inhibition of angiogenesis. In the dBP4-expressing subcutaneous tumours, expression of VEGF165 was abolished relative to controls.
Systemic administration of wildtype IGFBP 4 in female mice increased bone formation parameters such as serum osteocalcin and alkaline phosphatase as well as serum IGF I. This was not seen in mice administered a protease resistant IGFBP 4. The increase in IGF I bioavailability in mice administered wildtype IGFBP 4 was due to an IGFBP 4 protease-dependent pathway (Miyakoshi et al., 2001). That study did not elucidate the protease involved in the degradation of iGFBP 4. However, PAPP-A is the main protease involved in IGFBP 4 proteolysis and is expressed by osteoblasts (Lawrence et al., 1999) which could explain the increase in bone formation parameters in wildtype IGFBP 4 treated mice. Therefore, dBP4 should not increase bone formation as seen with the wildtype IGFBP 4 used by Miyakoshi et al (2001).

Bone metastasis is a common cause of morbidity in prostate cancer patients (Bubendorf et al., 2000) with limited therapies available. IGF I regulates tumour cell invasion via matrix metalloproteinases (MMPs) and uPA (Dunn et al., 2000). Up-regulation of the IGF IR increased MMP-2 expression, invasion and metastasis in H-59 cells, a subline of Lewis lung carcinoma cells relative to controls (Long et al., 1998). Therefore, blockade of IGF I and/or the IGF IR could reduce metastasis formation. Two models of metastasis were used in this study to generate bone and liver metastases. Intracardiac and IV injection of PC-3M-luc2 dBP4 cells did not give rise to a large number of mice with bone metastases. The liver was the main site of metastasis formation, probably because PC-3M cells are derived from a PC-3 liver metastasis (Kozlowski et al., 1984). dBP4-expressing liver metastases appeared to remain localised within the liver relative to controls where bioluminescence appeared diffuse throughout the liver when monitored using the IVIS imaging system. Due to time constraints, further analysis of the liver metastases were not carried out. No definitive conclusion could be drawn from the metastasis models possibly due to relatively small numbers in the intracardiac (n=3/group) and IV experimental metastasis groups (n=5/group). However, Jenkins et al., (2003) previously utilised PC-3M cells in an intracardiac model of bone metastasis with 5 mice per treatment group the same as the IV experimental metastasis model. In the intracardiac experimental metastasis model, after day 8 of dBP4 induction the BLI decreased in both the controls and dBP4-expressing mice. This suggests that the liver metastases became necrotic. Since the liver is the main source of serum IGF I within the body (Sjögren et al., 1999), dBP4 levels may not have been sufficient to inhibit IGF I leading to continued growth of PC-3M-luc2 dBP4-expressing liver metastases and no growth difference relative to controls. Colon adenocarcinoma tissue fragments were orthotopically transplanted to the surface of the caecum in control and liver-specific deficient (LID) mice in which serum IGF I levels are 25% of that in control mice (Wu et al., 2002). Mice were divided randomly into two groups; one group received recombinant human IGF I and the other group received saline. In the saline group, the incidence of hepatic metastasis...
was significantly higher in control mice relative to LID mice. Control or LID mice treated with recombinant human IGF I had significantly increased rates of liver metastases compared with saline-injected mice. The number of metastatic nodules in the liver was significantly higher in control mice relative to LID mice (Wu et al., 2002). This further supports the role of IGF I in the development of liver metastases.

Overall, dBP4 expression inhibited primary tumour growth in subcutaneous and orthotopic prostate cancer models. However, PC-3M-luc2 cells are PSA negative (Stephenson et al., 1992). PSA cleavage of dBP4 led to an increased number of fragments relative to cleavage of recombinant IGFBP 4. Therefore, mutation of the PAPP-A sequence may have introduced more cleavage sites for PSA due to either the change in amino acid sequence or a change in conformation. The PSA cleavage site for IGFBP 4 has not yet been established. However, the substrate specificity of PSA has been investigated, with tyrosine being the preferred site of hydrolysis. The consensus sequence SS(Y/F)Y\_SG was determined as the sequence for most efficient peptide hydrolysis by PSA (Coombs et al., 1998). Within IGFBP 4, there are 2 tyrosine residues, but the consensus sequence is not found in this protein (Appendix 13). Clinically, elevated PSA is indicative of prostate cancer (Fall et al., 2007). PSA is secreted by the luminal cells of the epithelium within the prostate (Peehl, 2006). Therefore, dBP4 may not be useful in prostate cancer patients with elevated PSA. However, < 1 % of men present with PSA-negative prostate carcinoma, which is defined as PSA <10 ng/ml (Lee and Oesterling, 1997). Patients presenting with treatment-naive PSA-negative metastatic prostate cancer showed similar characteristics to that of patients with high serum PSA levels, but their median survival and response to hormone therapy was much shorter than prostate cancer patients with high PSA levels (Birtle et al., 2003). Overall survival is poorer in patients with PSA-negative metastatic prostate cancer relative to patients with PSA-positive metastatic prostate cancer (Yamamoto et al., 2001). Even though a small percentage of patients present with PSA-negative prostate cancer, treatment of the disease has proved to be difficult. dBP4 may be useful in such cases where abnormally low PSA levels are seen.

Future studies should assess apoptosis and angiogenesis (CD31\(^*\) staining) in the subcutaneous and prostate tumours, since dBP4 inhibited IGF-induced microvascular endothelial cell proliferation in vitro (Ryan et al., 2009), dBP4 decreased IGF-induced tubule formation and dBP4 induced endothelial cell apoptosis in 4T1.2 breast cancer tumours suggesting an anti-angiogenic mechanism of action (Ryan et al., 2009). As this study suggested that locally expressed dBP4 either did not reach the systemic circulation or was not expressed at high enough levels to effect circulating IGF I, future studies with systemic administration of dBP4 should yield insight into the side effects of dBP4. In vitro, the PC-3M-luc2 cells expressed IGFBP 3, IGFBP 4 and produced low levels of IGF I. Therefore, dBP4
was probably blocking tumour and locally produced IGF I, but not circulating IGF I, most of which is produced by the liver. Kinome profiling of subcutaneous and prostate tumours may also give insight into alternative pathways that are inhibited or enhanced following dBP4 expression. To test the effect of dBP4 on bone metastasis intratibial injection of PC-3M-luc2 cells or use of orthotopic model with a different cell line may be a superior bone metastasis model. Systemic administration of dBP4 would reflect the clinical situation and would allow determination of the effect of dBP4 on tumour growth and side effects (hyperglycaemia) that may be associated with systemic administration of dBP4. Other future work may involve determining the PSA cleavage site in dBP4, to generate a PSA and PAPP-A protease resistant IGFBP 4. The effect of this protein could then be assessed using a PSA positive cell line such as LNCaP cells, which would reflect the majority of human prostate cancers.

dBP4 may prove useful as a neoadjuvant or adjuvant therapy to slow the growth of tumours, particularly tumours that are IGF I driven as dBP4 blocked IGF IR activation in vivo. Inhibition of the IGF pathway using dBP4 proved effective in inhibiting tumour growth in vivo in this study. However, resistance pathways can occur following long-term treatment with most cancer treatments (Chen et al., 2004, Desbois-Mouthon et al., 2009, Garofalo et al., 2011, Kute et al., 2004). In PC-3M-luc2 cells, the IGF IR was shown to form heterodimers with the EGFR; therefore, future studies may include using dBP4 in combination with an EGFR inhibitor such as Lapatinib (Glaxo Smith Kline).

Frequent re-evaluation of response and changing treatment when necessary analogous to HIV treatments where therapy is changed when CD4+ count increases may be beneficial in cancer treatment regimes. Therefore, reliable serum biomarkers to indicate tumour re-growth or progression are needed. Moreover, molecular profiling of tumours now gives information on tumour status and predicts response to therapy, thus allowing each individual patient to receive a specific drug regime based on tumour type and expression of genes or proteins (Diamandis et al., 2010, Garman et al., 2007, Malinowsky et al., 2011). There is now a general acceptance that eradicating all cancer cells is probably impossible with any therapy and achieving disease stabilisation is a more realistic goal (Kelly, 2003). Preventing cancer progression would increase patient survival and make cancer a manageable disease rather than a curable one.
References


Greenberg, N. M., Demayo, F., Finegold, M. J., Medina, D., Tilley, W. D., Aspinall, J. O.,
in a transgenic mouse. Proceedings of the National Academy of Sciences, 92, 3439-
3443.

Greenberg, N. M., Demayo, F. J., Sheppard, P. C., Barrios, R., Lebovitz, R., Finegold, M.,
probasin gene promoter directs hormonally and developmentally regulated
expression of a heterologous gene specifically to the prostate in transgenic mice.
Molecular Endocrinology, 8, 230-239.

Receptor Stabilization in Recurrent Prostate Cancer Is Associated with

Greiner, D. L., Shultz L.D., Yates, J., Appel, M. C., Perdrizet, G., Hesselton R.M.,
engraftment of human spleen cells in NOD/LtSz-scid/scid mice as compared with
C.B-17-scid/scid mice. American Journal of Pathology, 146, 888-902.

Gupta, S., Adhami, V. M., Subbarayan, M., Maclennan, G. T., Lewin, J. S., Hafeli, U. O., Fu,
Supplementation of Celecoxib in Transgenic Adenocarcinoma of the Mouse Prostate
Model. Cancer Research, 64, 3334-3343.

Habuchi, T., Suzuki, T., Sasaki, R., Wang, L., Sato, K., Satoh, S., Akao, T., Tsuchiya, N.,
Association of Vitamin D Receptor Gene Polymorphism with Prostate Cancer and
Benign Prostatic Hyperplasia in a Japanese Population. Cancer Research, 60, 305-
308.

Promote Osteoblastic Bone Metastases through Wnts. Cancer Research, 65, 7554-
7560.

Epidermal growth factor—induced activation of the insulin-like growth factor I
receptor in rat hepatocytes. Hepatology, 36, 1509-1518.

Haluska, P., Shaw, H. M., Batzel, G. N., Yin, D., Molina, J. R., Molife, L. R., Yap, T. A.,
I Dose Escalation Study of the Anti–Insulin-Like Growth Factor-I Receptor
Monoclonal Antibody CP-751,871 in Patients with Refractory Solid Tumors. Clinical
Cancer Research, 13, 5834-5840.


Tennant, M., Thrasher, J., Twomey, P., Drivdahl, R., Birnbaum, R. & Plymate, S. 1996a. Protein and messenger ribonucleic acid (mRNA) for the type 1 insulin-like growth factor (IGF) receptor is decreased and IGF-II mRNA is increased in human prostate carcinoma compared to benign prostate epithelium. *Journal of Clinical Endocrinology & Metabolism*, 81, 3774-3782.


Youngren, J., Gable, K., Penaranda, C., Maddux, B., Zavodovskaya, M., Lobo, M., Campbell, M., Kerner, J. & Goldfine, I. 2005. Nordihydroguaiaretic Acid (NDGA)


Bibliography


Websites


Appendices
Appendix 1: Vector map of pTriEx-Neo

Image from Novagen (www.merck-chemicals.se)
Appendix 2: Vector map of pTet On Advanced

![Vector map of pTet On Advanced](image)

Image taken from [www.clontech.com](http://www.clontech.com)
Appendix 3: Vector map of pLVX Tight Puro

Image taken from www.clontech.com
Appendix 4: Vector map for psPAX2

Taken from www.addgene.org/12260/
Appendix 5: Vector map for pmD2.g

Image taken from www.addgene.org/12259/
# Appendix 6: Materials and buffers for Western blot, FPLC and DNA electrophoresis

## 6 x Laemml Buffer
To prepare 10 ml:
- Trisma Base 6.06 g
- SDS 1.3 g
- Glycerol 4.7 ml
- Bromophenol blue 0.6 g
- DTT 0.93 g
- dH₂O 2.1 ml

## Electrode buffer 500 ml
- Trisma Base 6.06 g
- HEPES 11.9 g
- SDS 8.64 g
dH₂O up to 500 ml

## 10 x Transfer Buffer
- Trisma Base 21.7 g
- Glycine 112.6 g
- SDS 1 g
dH₂O up to 1 L

## Working Transfer Buffer
- 10 x Transfer buffer 100 ml
- 100 % Methanol 200 ml
dH₂O 700 ml

## 10 x TBS
- Trisma Base 30.27 g
- NaCl 87.66 g
dH₂O 900 ml
Adjust to pH 7.6 with HCl
Adjust to 1 L with dH₂O

## Wash Buffer 1 x TBST
- 10 x TBS 100 ml
- Tween 1 ml
dH₂O 899 ml

## Blocking Buffer
- 1 x TBST 50 ml
- Marvel fat free milk 2.5 g
Or
- BSA 2.5 g
- SDS 0.1 g
dH₂O up to 100 ml

## RIPA Buffer (100 ml)
- Tris HCl 0.39 g
- NaCl 0.12 g
NP-40 1 ml
Sodium deoxycholate 1 g
### FPLC Running Buffer
- HEPES: 2.38 g
- NaCl: 12.3 g
- CaCl\(_2\): 0.01 g
- pH 7.5
  - dH\(_2\)O make up to 1L

### 6 x DNA loading buffer (10 ml)
- Bromophenol blue: 0.025 g
- Xylene cyanol FF: 0.025 g
- Glycerol: 3.3 ml
  - dH\(_2\)O make up to 10 ml

### 1 x TAE Buffer
- Tris: 4.84 g
- Acetic Acid: 1.2 g
- EDTA: 0.29 g
  - dH\(_2\)O make up to 1 L
Appendix 7: Representative protein assay standard curve

The graph shows a standard curve for the Bio Rad DC protein assay. The equation of the line is:

$y = 0.129x + 0.0059$

with $R^2 = 0.9972$. The x-axis represents protein concentration (mg/ml) and the y-axis represents absorbance at 750 nm.
## Appendix 8: Antibodies used for IHC, IP and Western blotting

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>APPLICATION WB/IP/IHC</th>
<th>BLOCK</th>
<th>PRIMARY SOURCE, DILUTION, BUFFER</th>
<th>SECONDARY SOURCE, DILUTION, BUFFER</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP 4</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Millipore (MA, USA), rabbit anti-human/mouse IGFBP4 1:4000 in 5% marvel in TBST (0.1%), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP goat anti-rabbit 1:2000 in 5% marvel TBST (0.1%), incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>IGF1R β</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Cell Signalling Technology (MA, USA), rabbit anti-human/mouse, 1:1000 in 5% BSA TBST (0.1% Tween), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:2000 in 5% marvel TBST (0.1%), incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>pIGF1R (Tyr 1131)</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Invitrogen, rabbit anti-human, 1:2000 in 5% marvel TBST (0.1% Tween), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:2000 in 5% marvel TBST (0.1%), incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>Akt</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Cell Signalling Technology (MA, USA), rabbit anti-human/mouse, 1:2000 in 5% BSA TBST (0.1% Tween), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:2000 in 5% marvel TBST (0.1%), incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>pAkt (Ser 473)</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Cell Signalling Technology (MA, USA), monoclonal mouse anti-human/mouse, 1:2000 in 5% Milk TBST (0.1% Tween), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-mouse 1:5000 in 5% marvel TBST (0.1%), incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>IGFBP 3</td>
<td>WB</td>
<td>3% Marvel in PBST (0.05% Tween)</td>
<td>Millipore (MA, USA), rabbit anti human/mouse 1:1000 in 3% marvel PBST (0.05% Tween), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:2000 in 3% marvel PBST (0.05%), incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>EGFR</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Cell signalling Technology (MA, USA), rabbit anti-human/mouse 1:2000 in 5% BSA TBST (0.01% Tween), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:2000 in 5% marvel TBST (0.1%), incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>Antibody</td>
<td>Type</td>
<td>Dilution</td>
<td>Source</td>
<td>Buffer</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>----------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>pEGFR (Tyr 1173)</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Cell signalling Technology (MA, USA) rabbit monoclonal anti-human/mouse, 1:2000 in 5% BSA in TBST (0.1% Tween) incubated overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:2000 in 5% marvel TBST (0.1%), Incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>pEGFR (Tyr 1173)</td>
<td>IP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin R</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Cell Signalling technology (MA, USA) mouse monoclonal anti-human/mouse, 1:1000 in 5% marvel in TBST (0.1% Tween) incubated overnight at 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-mouse 1:2000 in 5% marvel TBST (0.1%), Incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>pInsulin R (Tyr 1185)</td>
<td>WB</td>
<td>5% Marvel in TBST (0.1% Tween)</td>
<td>Cell Signalling technology (MA, USA) mouse monoclonal anti-human/mouse, 1:1000 in 5% marvel in TBST (0.1% Tween) incubated overnight at 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-mouse 1:2000 in 5% marvel TBST (0.1%), Incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>Insulin R</td>
<td>IP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pInsulin R (Tyr 1185)</td>
<td>IP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF1R β</td>
<td>IP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIGFIR (Tyr 1131)</td>
<td>IP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

251
<table>
<thead>
<tr>
<th>Protein</th>
<th>Method</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1R β</td>
<td>IHC</td>
<td>Cell Signalling technology (MA, USA), rabbit anti-human/mouse 1:100</td>
<td>No secondary, as primary is HRP conjugated</td>
<td></td>
</tr>
<tr>
<td>His-tag</td>
<td>WB</td>
<td>Genscript (NY, USA), 1:10000 in 5% marvel TBST (0.1% tween), overnight @ 4°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>WB</td>
<td>Abcam (MA, USA), rabbit anti-human 1:5000 in 5% marvel in TBST (0.1% tween), overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) anti-rabbit HRP 1:2000 in 5% marvel in TBST (0.1% tween) incubate @ room temp for 1 hr</td>
<td></td>
</tr>
<tr>
<td>ERK p44/42</td>
<td>WB</td>
<td>Cell signalling Technology (MA, USA) rabbit anti-human/mouse, rat 1:1000 in 5% BSA in TBST (0.1% tween), incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:5000 in 5% marvel in TBST (0.1%), Incubate @ room temp for 1 hr</td>
<td></td>
</tr>
<tr>
<td>ERK p44/42 Thr202/Tyr204</td>
<td>WB</td>
<td>Cell signalling Technology (MA, USA) rabbit anti-human/mouse, rat 1:1000 in 5% BSA in TBST (0.1% tween), incubate</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:5000 in 5% marvel in TBST (0.1%), Incubate @ room temp for 1 hr</td>
<td></td>
</tr>
<tr>
<td>α-tubulin (clone DM1A)</td>
<td>WB</td>
<td>Sigma Aldrich (MO, USA) mouse anti-human, rat 1:5000 in 5% Marvel in TBST (0.1% tween). Incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-mouse 1:2000 in 5% marvel TBST (0.1%). Incubate @ room temp for 1 hr</td>
<td></td>
</tr>
<tr>
<td>rtTA</td>
<td>WB</td>
<td>5 % Marvel in TBST (0.1 % tween)</td>
<td>Clontech (CA, USA) mouse anti-human 1:500 in 5 % Marvel in TBST (0.1 % tween). Incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-mouse 1:2000 in 5 % marvel TBST (0.1 %). Incubate @ room temp for 1 hr</td>
</tr>
<tr>
<td>------</td>
<td>----</td>
<td>-------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>VEGF</td>
<td>WB</td>
<td>5 % Marvel in TBST (0.1 % tween)</td>
<td>Santa Cruz (MA, USA) rabbit anti-human 1:250 in 5 % Marvel in TBST (0.1 % tween). Incubate overnight @ 4°C</td>
<td>DAKO (Glostrup, Denmark) HRP anti-rabbit 1:2000 in 5 % marvel TBST (0.1 %). Incubate @ room temp for 1 hr</td>
</tr>
</tbody>
</table>

**WB= Western blotting**  
**IP= immunoprecipitation**  
**IHC= immunohistochemistry**
Appendix 9: Representative VEGF$_{165}$ standard curve

![VEGF Standard Curve](image-url)

- $y = 0.0022x + 0.0652$
- $R^2 = 0.9967$
Appendix 10: Representative human IGF I standard curve

Plasma IGF I Standard Curve

\[ y = 0.3325x - 0.0415 \]
\[ R^2 = 0.9975 \]
Appendix 11: Representative mouse IGF standard curve

Mouse IGF I standard curve:

\[ y = 0.0012x + 0.1049 \]

\[ R^2 = 0.9851 \]
Appendix 12: Representative human PAPP-A standard curve
Appendix 13: Amino acid sequence of protease resistant IGFBP 4

MVPFGLVAALLLAAGPRPSLGEAIHCPCSEEEKLARCPPVGCCEELVREPCCGCATCALGLGMPCGYTPRCGSGMRCYPPRGV
VEKPLRTLHMQGVCTELSIEIAIQESLQTSKDSEHPNNSF
NPCSAHDHRCLQAAAMAAVADASAMAIVGTPREEPRPVPQGSC
QSELHRAELAASQSRTHDELFIIPNPCDRNGNFHPKQCHPA
LDGQRGKCWCVDRTGVKLPGLPKGELDCHQLADSFQEDD
DDKSLEHHHHHHHH

\textbf{MVPFGLVAALLLAAGPRPSL}G = \text{Signal peptide}

\textbf{AAAMAAVADASAMA} = \text{mutated PAPP-A cleavage site}

\textbf{DDDDKSLE} = \text{enterokinase}

\textbf{HHHHHHHH} = \text{His-tag}
Appendix 14: Presentations and awards

Selected for the EMBO Short term travel scholarship April 2011
To travel to Dana Farber Cancer Institute for 2 months.

RCSI Research Day, May 2011
Abstract

RCSI Research Day, April 2010
Poster Presentation

IACR, Galway, March 2010
Poster presentation

RCSI Research Day, March 2009
Poster Presentation

Prostate Cancer Research Consortium Meeting Dec 2009
Oral Presentation