Intercellular Signalling in the Prostate Cancer Tumour Microenvironment: Implications for Disease Progression



Kristie McCormick

Balliol College

University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy

Department of Physiology, Anatomy and Genetics

Michaelmas 2018

i. Acknowledgements

Firstly, I would like to thank my supervisor, Deborah Goberdhan, for providing me the opportunity to work in her lab. I appreciate the time she has dedicated to me these past four years and the freedom she has given me to explore my own ideas during my DPhil. I would also like to thank Clive Wilson for offering his advice and insight throughout my doctoral studies, and more recently, for helping me with my decision to pursue a career in medicine.

I am grateful to all members of the Goberdhan lab, past and present, for their support. In particular, I would like to express my immense gratitude to Shih-Jung Fan for all of the guidance he has offered me during my DPhil. Learning from him has been a humbling experience: his hardworking nature and rigorous approach to research is unmatched, and I wish him every success in his career – no one deserves it more. I am also immeasurably thankful to Irina Stefana for her mentorship and friendship. From the hours she dedicated to teaching me confocal microscopy, to the long afternoons she spent discussing my work – her generosity knows no bounds, and I am forever grateful that I had the opportunity to learn from someone so insightful with such a passion for science. I owe special thanks to Cleidson Alves for passing on his expert knowledge of molecular biology, and for exposing me to the lunchtime offerings of Cosmo. I am especially grateful to newcomer Matt Ellis for dedicating so much of his first year to teaching me cloning techniques, and to John Mason, for sharing his vast clinical knowledge with me (and pictures of his beautiful baby, Jasper!). I would like to thank them both for their friendship and for filling my final year with so much laughter. I am also grateful to Kate Carr for her advice and feedback; to Hakimi Kassim for his helpful input in lab meetings; and to my student, Anusha Sabanayagam, for her help in optimising the exosome isolation protocol during the first year of my DPhil, and for her friendship since.

In addition, I would like to thank the Wilson lab for sharing their research with us over the years, and for opening my eyes to the amazing work that can be achieved using *Drosophila*! I am particularly grateful to Ben Kroeger, Josephine Hellberg, Mark Wainwright, and Aashika Sakar and for their help with confocal microscopy on numerous occasions. I am also grateful for the friendships I have formed with numerous members of the Wilson lab. I am particularly thankful to Josephine Hellberg and Ben Kroeger for their friendship outside of the lab, and for filling my DPhil experience with so many wonderful memories.

I am enormously grateful to several members of the Wood lab for offering their technical help and advice over the years. In particular, I would like to express my thanks to Eduard Wilms for sharing his impressive knowledge of exosome isolation protocols. I am also grateful to Carlo Rinaldi for helping me optimise the luciferase assays presented in this thesis, and to Anja Kruger, for generously dedicating many afternoons to teaching me immuno-EM and for the many evenings we spent sharing a glass of wine.

I would also like to thank John Morris and Errin Johnson for sharing their expertise in electron microscopy - learning from them both was a real privilege.

I am extremely appreciative of Dan Stevens, Aaron Leiblich and our clinical collaborators, Richard Bryant and Freddie Hamdy, for offering their unique insight into the clinical aspects of this project. I would like to thank Freddie in particular for his support throughout my DPhil, and for his encouragement in beginning this next chapter in my life.

Furthermore, I am of course very thankful to both my parents for their unwavering support, and for never once questioning my perpetual student status!

Finally, to my partner, Sam: thank you for being a continual source of love and encouragement these past four years. Whenever life in the lab became particularly challenging, I could always count on your support to help me through. I am so lucky to have you by my side.

ii. <u>Abstract</u>: Intercellular signalling in the prostate cancer tumour microenvironment: implications for disease progression

D.Phil. in Physiology, Anatomy and Genetics, Michaelmas Term, 2018

Advanced prostate cancer (PCa) is incurable and presents a significant clinical challenge. Once metastasised, crosstalk between PCa cells and other cells within the tumour microenvironment (TME) is critical for tumour growth. Hence, disrupting tumour-stroma interactions has been highlighted as a therapeutic strategy to combat disease progression. However, the intercellular signalling mechanisms underpinning PCa cell growth within the TME are still incompletely understood. Enhanced signalling via the bone morphogenetic protein-6 (BMP-6) and mammalian target of rapamycin complex 1 (mTORC1) pathways is frequently observed in advanced PCa tumours. Whilst the role of BMP-6 in regulating PCa cell growth remains an area of intense debate, the growth-promoting effects of mTORC1 is well-established. Nonetheless, targeting mTORC1 has had limited clinical success in PCa, hence further understanding of the upstream and downstream mechanisms by which mTORC1 regulates cell growth is crucial. Therefore, this thesis focuses on understanding the ways in which these two major growth factor signalling pathways regulate tumour growth and the implications for devising future therapeutic strategies.

Here, I characterise the effect of BMP-6 on the growth of several PCa cell lines and identify a role for the androgen receptor (AR) and retinoblastoma (Rb) in mediating the growth-suppressive effects of BMP-6. I show that treatment with BMP-6 inhibits AR activity, Rb phosphorylation and cell growth in the androgen-responsive LNCaP cell line, but not in the androgen-insensitive derivative cell line C4-2B. Furthermore, I propose that alterations in this pathway enables cells to escape the growth-inhibitory effects of BMP-6 in advanced PCa and provide support for this model using existing clinical data. I also examine the effects of altered mTORC1 signalling on the secretion of extracellular vesicles (EVs) - membrane-bound intercellular messengers that carry multiple molecular cargoes and can reprogram target cell behaviour. I show that decreased mTORC1 activity alters the type of EVs released from two different PCa cell lines in a manner reminiscent of an event recently characterised by our lab in colorectal cancer cells, termed an ‘exosome switch’, and that these EVs alter the growth of recipient PCa cells *in vitro*. Furthermore, I reveal that androgen-deprivation represents another form of microenvironmental stress that regulates a similar event in PCa cells and identify increased secretion of AR-positive EVs as a novel characteristic of the switching response. Furthermore, I provide evidence to suggest that these ‘switched’ EVs include a heterogeneous population of vesicles consisting of at least two different EV subtypes. Finally, I show that upstream of mTORC1, the amino acid transporter PAT4 acts, in part, to mediate the stimulatory effects of androgens on mTORC1 activity and PCa cell growth, and I propose a further role for PAT4 in regulating the exosome switching event in PCa.

In summary, my research reveals novel ways in which signalling via the BMP-6 and mTORC1 pathways regulate PCa cell growth and highlights potential clinical targets and therapeutic avenues for the treatment of patients with advanced PCa.

iii. List of Abbreviations

Abbreviation	Description
4E-BP1	Eukaryotic translational initiation factor **4E**-**b**inding **p**rotein **1**
ADT	**A**ndrogen **d**eprivation **t**herapy
Alix	**AL**G-2-**i**nteracting protein **X**
AR	**A**ndrogen **r**eceptor
ARE	**A**ndrogen **r**esponse **e**lement
AR-V	**A**ndrogen **r**eceptor **s**plice **v**ariant
BCR	**B**iochemical **r**ecurrence
BM	**B**one **m**arrow
BMP	**B**one **M**orphogenetic **P**rotein
BSA	**B**ovine **s**erum **a**lbumin
CHMP	**Ch**arged **M**ultivesicular body **p**rotein
CRPC	**C**astration-**r**esistant **p**rostate **c**ancer
CSS	**C**harcoal-**s**tripped **s**erum
Deptor	**DEP** Domain Containing M**TOR**-Interacting Protein
DHT	**D**i**h**ydro**t**estosterone
DMSO	**D**i**m**ethyl **s**ulf**o**xide
DNA	**D**eoxyribo**n**ucleic **A**cid
DRE	**D**igital **r**ectal **e**xam
dUC	**D**ifferential **U**ltra**c**entrifugation
EE	**E**arly **e**ndosome
ESCRT	**E**ndosomal **s**orting **c**omplex **r**equired for **t**ransport
EV	**E**xtracellular **v**esicles
FBS	**F**oetal **b**ovine **s**erum
GAPs	**G**TPase-**a**ctivating **p**roteins
GEF	**G**uanine **n**ucleotide **e**xchange **f**actor
Gln	L-**Gl**utami**n**e
GR	**G**lucocorticoid **r**eceptor
GTP	**G**uanosine-5'-**t**ri**p**hosphate
ILV	**I**ntra**l**uminal **v**esicle

ITS	**I**nsulin-**t**ransferrin-**s**elenium
LAMP-2	**L**ysosomal-**a**ssociated **m**embrane **p**rotein-2
LBD	**L**igand-**b**inding **d**omain
LELs	**L**ate **e**ndosome and **l**ysosomes
LEs	**L**ate **e**ndosomes
MeOH	**Me**thanol
miRNAs	**M**icro-**r**ibo**n**ucleic **a**cid
mLST8	**M**ammalian **l**ethal with **s**ec-13 protein **8**
mRNA	**M**essenger **RNA**
mTORC1/2	**M**echanistic **t**arget **o**f **r**apamycin **c**omplex **1/2**
MVE	**M**ultivesicular **e**ndosome
NTA	**N**anoparticle **t**racking **a**nalysis
NTD	**N**-**t**erminal **d**omain
PAT	**P**roton **a**ssisted **t**ransporter
PCa	**P**rostate **c**ancer
PBS	**P**hosphate-**b**uffered **s**aline
PCR	**P**olymerase **c**hain **r**eaction
PI3K	**P**hosphatidyl**i**nositol-**3**-**k**inase
PIKK	**P**hosphatidyl**i**nositol 3-**k**inase-related **k**inase
PM	**P**lasma **m**embrane
PRAS40	**P**roline-**r**ich **A**kt **s**ubstrate **40**kDa
Protor	**Pr**otein **o**bserved with ric**TOR**
PSA	**P**rostate-**s**pecific **a**ntigen
Raptor	**R**egulatory-**a**ssociated **p**rotein of m**TOR**
Rb	**R**etino**b**lastoma
RE	**R**ecycling **e**ndosome
Rheb	**R**as **h**omolog **e**nriched in **b**rain
rictor	**R**apamycin-**i**nsensitive **c**ompanion of m**TOR**
RNA	**R**ibo**n**ucleic **a**cid
RP	**R**adical **p**rostatectomy
RT	**R**adio**t**herapy
S6K	Ribosomal protein **S6 k**inase

SEC	**S**ize **e**xclusion **c**hromatography
Sin1	**S**tress-activated protein kinase-**in**teracting protein **1**
SLC	**S**olute-**l**inked **c**arrier
TEM	**T**ransmission **e**lectron **m**icroscopy
TGF-β	**T**ransforming **g**rowth **f**actor-**β**
TFF	**T**angential **f**low **f**iltration
TGN	**T**rans-**G**olgi **n**etwork
TME	**T**umour **m**icro**e**nvironment
TSC1/TSC2	**T**uberous **S**clerosis **c**omplex **1/2**
Tsg101	**T**umour **s**usceptibility **g**ene **101**
UC	**U**ltra**c**entrifugation
UF	**U**ltra**f**iltration
UV	**U**ltra**v**iolet
VPS	**V**acuolar **s**orting **p**rotein

iv. Contents

i. Acknowledgements……...1

ii. Abstract……....2

iii. List of Abbreviations……3

v. List of Figures……..……..10

vi. List of Tables……..……..11

1. Introduction 13

1.1 Prostate cancer 13

1.1.1 Incidence and mortality 13

1.1.2 Screening 14

1.1.3 Diagnosis 15

1.1.4 Management 16

1.1.5 Prognosis 21

1.2 Challenges in treating advanced PCa 21

1.2.1 The role of androgens in the prostate 21

1.2.2 The AR signalling pathway 22

1.2.3 AR signalling in advanced PCa 22

1.2.4 Mechanisms underlying CRPC 25

1.3 The tumour microenvironment (TME) 29

1.3.1 The TME in advanced PCa 29

1.3.2 Extracellular signals in the TME: BMPs 30

1.3.3 Extracellular signals in the TME: nutrients 35

1.3.4 Extracellular signals in the TME: membrane-bound vesicles 42

1.4 Aims and objectives 55

2.1 Materials and Methods 58

2.1.1 Prostate cancer cell characterisation 58

2.1.1.1 Cell culture 58

2.1.1.2 Genetic manipulation of PCa cell lines 61

2.1.2 Isolation and characterisation of EVs 64

2.1.2.1 Isolation conditions 64

2.1.2.2 EV isolation 64

2.1.2.3 EV characterisation 67

2.1.3 Protein and RNA isolation and analysis 71

2.1.3.1 Western blot analysis 71

2.1.3.2 Quantitative RT–PCR 73
2.1.4 Analysis of BMP-6 treatment on PCa cell behaviour 74
2.1.4.1 Proliferation assays 74
2.1.4.2 Cell growth assays 74
2.1.4.3 Co-culture assays 74
2.1.4.4 Luciferase assay 76
2.1.4.5 Immunofluorescence 76
2.1.5 Statistical analysis 77

3. The role of BMP-6 signalling in the regulation of PCa cell growth 79
3.1. Introduction 79
3.1.1 Function and expression of BMPs in PCa 79
3.1.2 Aims and objectives 81
3.2 Results 82
3.2.1 Establishing conditions to examine the effects of BMP-6 on PCa cell growth 82
3.2.2 Characterising the effects of BMP-6 on the growth of a panel of PCa cell lines 85
3.2.3 Investigating how the bone microenvironment affects PCa cell growth 89
3.2.4 Confirming differences in androgen sensitivity between the PCa cell lines 94
3.2.5 Examining mechanisms underlying PCa cell growth 96
3.2.5.1 Crosstalk with AR signalling 96
3.2.5.2 Cell cycle control 102
3.2.5.3 Regulation of Rb 104
3.3. Discussion 107
3.3.1 BMP-6 can affect the growth of certain PCa cell lines 107
3.3.2 The bone environment mimics the effects of BMP-6 on PCa cell growth 108
3.3.3 AR may be involved in regulating the growth-suppressive effects of BMP-6 109
3.3.4 BMP-6-induced growth inhibition is partially mediated by Rb 110
3.3.5 Clinical implications 113

4. Investigating the role of the PAT4-mTORC1 signalling axis in PCa 119
4.1. Introduction 119
4.1.1 Amino acid sensing in the cell 119
4.1.2 Amino acid sensing and the PATs 120
4.1.3 The multi-hub model of mTORC1 signalling 121
4.1.4 The PAT4-mTORC1 axis regulates exosome signalling in colorectal cancer cells 121

4.1.5 PAT4 expression and EV signalling are altered in PCa 122
4.1.6 Aims and objectives 122
4.2 Results 123
4.2.1 PAT4 regulates mTORC1 signalling in PCa cells 123
4.2.2 PAT4 expression is regulated by androgens 125
4.2.3 Androgens promote PAT4-mediated PCa cell growth 129
4.2.4 Establishing media conditions to isolate EVs from PCa cells 131
4.2.5 Characterising PCa EVs isolated via size exclusion chromatography (SEC) 134
4.2.6 Glutamine starvation induces EV switching in LNCaP cells 137
4.2.7 mTORC1 inhibition induces EV switching in LNCaP and C4-2B cells 143
4.3. Discussion 147
4.3.1 Androgens promote PAT4 expression 147
4.3.2 DHT-induced mTORC1 activation and cell growth is partially mediated by PAT4 149
4.3.3 SEC is an effective method to isolate EVs from PCa cell culture media 151
4.3.4 The mTORC1 pathway regulates EV signalling in PCa cells 153
4.3.5 Clinical implications 154

5. Exploring the regulation of EV signalling by microenvironmental stress in PCa 159
5.1. Introduction 159
5.1.1 EV heterogeneity and function in cancer 159
5.1.2 Current issues in the development of EV-based therapeutics 160
5.2 Results 162
5.2.1 Establishing conditions to isolate EVs from cells treated with anti-androgens 162
5.2.2 Validating the EV isolation protocol 166
5.2.3 Androgen-deprivation induces EV switching in VCaP cells 170
5.2.4 mTORC1 inhibition induces AR secretion in LNCaP cells 172
5.2.5 AR released by LNCaP cells is associated with EVs 174
5.2.6 AR released by LNCaP cells is functional 177
5.2.7 Inhibition of mTORC1 or AR signalling affects a heterogeneous EV population 179
5.3. Discussion 184
5.3.1 The AR pathway regulates EV signalling in PCa cells 185
5.3.2 The AR marks a heterogeneous population of EVs 186
5.3.3 The AR and mTORC1 pathways regulate at least two distinct EV populations 189
5.3.4 Clinical implications 190

6. Concluding remarks 195
6.1 Summary of research aims 195
6.1.1 BMP-6 inhibits the growth of androgen-sensitive PCa cells (i) 196
6.1.2 AR and Rb may mediate the growth-inhibitory effects of BMP-6 (i) 197
6.1.3 PAT4 regulates mTORC1 activity and PCa cell growth (ii) 198
6.1.4 mTORC1 signalling regulates EV signalling in PCa cells (ii) 200
6.1.5 Microenvironmental stress promotes exosome switching in PCa cells (iii) 200
6.1.6 Switched EVs include a heterogeneous population of EVs (iii) 201

7. References 203

vi. List of Figures

1.1. Stages of PCa 17
1.2. Overview of the AR signalling pathway 23
1.3. Targets of androgen ablation in PCa 24
1.4. Mechanisms of CRPC 26
1.5. Overview of the canonical BMP signalling pathway 32
1.6. Components of mTORC1 and mTORC2 37
1.7. Overview of mTORC1 activation 39
1.8. PAT4 protein expression in prostatic tissue 42
1.9. The endocytic pathway 45
1.10. ESCRT-dependent ILV formation 47
1.11. EV heterogeneity 51

2.1. The main strategies used for EV isolation and characterisation 66

3.1. BMP-6 inhibits LNCaP cell proliferation in a wide range of culture conditions 83
3.2. BMP-6 can differentially affect PCa cell growth 87
3.3. BMP-6 inhibits LNCaP, but not PC-3, cell proliferation 88
3.4. Direct co-culture with bone marrow stromal cells mimics the effect of BMP-6 on LNCaP and PC-3 cell proliferation 90
3.5. Indirect co-culture with bone marrow stromal cells mimics the effect of BMP-6 on the growth of LNCaP, PC-3 and C4-2B cells 92
3.6. Characterisation of AR expression and androgen sensitivity profiles of different PCa cell lines 95
3.7. BMP-6 increases AR protein levels in LNCaP and C4-2B cells 97
3.8. BMP-6 does not affect the localization of AR in LNCaP or C4-2B cells 99
3.9. BMP-6 represses the transactivation of AR in LNCaP and HEK293 cells, but not C4-2B cells 100
3.10. BMP-6 promotes a molecular profile reflective of cell cycle inhibition in LNCaP, but not C4-2B, cells 103
3.11. Rb loss diminishes the growth-inhibitory effect of BMP-6 in LNCaP cells 105
3.12. Speculative model to explain metastatic dormancy and reactivation of PCa tumour cells in the bone 117

4.1. Establishing conditions to knockdown PAT4 in VCaP cells 124
4.2. DHT stimulates PAT4 expression in VCaP cells 129
4.3. PAT4 knockdown inhibits VCaP cell growth and mTORC1 activity 132

4.4. Establishing media conditions for EV isolation from LNCaP and C4-2B cells..... 134
4.5. Characterisation of EVs isolated from LNCaP and C4-2B cells..... 137
4.6. Examining the sensitivity of LNCaP and C4-2B cells to glutamine depletion and rapamycin treatment..... 140
4.7. Glutamine depletion alters EV signalling in LNCaP, but not C4-2B, cells..... 142
4.8. Assessing the sensitivity of LNCaP and C4-2B cells to Torin1 treatment..... 145
4.9. mTORC1 inhibition alters EV signalling in both LNCaP and C4-2B cells 146

5.1. Establishing media conditions to isolate EVs from VCaP and LNCaP cells following anti-androgen treatment..... 165
5.2. Characterisation of EVs isolated from VCaP cells and culture medium..... 169
5.3. Conditions of mTORC1-mediated microenvironmental stress enhance the secretion of AR from PCa cells..... 172
5.4. The AR in LNCaP conditioned medium is membrane-bound and marks a heterogeneous population of EVs..... 176
5.5. EVs derived from mTORC1-inhibited LNCaP cells alters PCa cell growth 179
5.6. PCa cells secrete a heterogeneous population of EVs 183-184

v. List of Tables

2.1. Overview of PCa cell lines used in this study..... 58
2.2. List of plasmids used in this study 63
2.3. List of antibodies used for western blotting 72
2.4. List of primer sequences used for qRT-PCR..... 73

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Prostate cancer

1.1.1 Incidence and mortality

Prostate cancer (PCa) is a leading cause of mortality and morbidity among men worldwide (Ferlay et al., 2013). In the UK, PCa is the most frequently diagnosed malignancy in men, and, after lung cancer, the second most common cause of cancer-related deaths (Cancer Research UK, 2016). Several published reports have revealed that the incidence of PCa has grown considerably in recent decades (Cook et al., 2015; Somdyala et al., 2015; Feletto et al., 2015). Two different theories have been proposed to explain this observed trend. The first suggests that increased PCa detection, following the widespread introduction of interventional screening, is responsible. Indeed, the rapid implementation of diagnostic testing using prostate-specific antigen (PSA) in the mid- to late1980s saw the age-adjusted incidence rate of PCa in the United States double by the early 1990s (Hankey et al, 1999); a similar trend was reported in several other high-income countries thereafter (Etzioni et al., 2002; Kvale et al., 2007; Larrañaga et al., 2010). However, in some countries, even though the uptake of PSA testing was considerably slower, an increase in the incidence rate of PCa was still observed (Williams et al., 2011; Hamoen et al., 2013), suggesting additional factors may be involved.

The second explanation proposes that incidence rates are rising due to the increasing prevalence of risk factors associated with PCa. The etiopathology of PCa has been linked to diet, obesity, smoking and environmental influences (Crowe et al., 2008; Pischon et al., 2008; Huncharek et al., 2010; Ferrís-i-Tortajada et al., 2011), however, so far, the only three firmly-established risk factors include age, race and family history (Leitzmann and Rohrmann, 2012). Whilst several studies have demonstrated that an individual's ethnicity

and family history are both significant factors in determining one's risk of developing PCa (Johns and Houlston, 2003; Metcalfe et al., 2008; Ben-Shlomo et al., 2008), arguably the greatest risk factor is age. Approximately 85% of all PCa cases are diagnosed in men aged over 65, whereas the reported incidence in those younger than 50 is estimated be 0.1% (Patel and Klein, 2009). Moreover, extrapolation of data obtained from autopsy studies has revealed that most men would likely develop PCa if they lived long enough (Gronberg et al., 2003). Considering the age and life expectancy of the global population is increasing (World Health Organisation, 2011), it is therefore likely that so too will the incidence rate of PCa (Wong et al., 2016).

Overall, it has been estimated that by 2030 there will be over 20 million new PCa patients; of which, approximately 65% (~13.2 million) will not survive (Hassanipour-Azgomiet et al., 2016). Globally, this will undoubtedly place a huge burden on both the individuals affected as well as society as a whole. Therefore, new and innovative therapeutic strategies are needed to improve the clinical outcomes for current and future PCa patients.

1.1.2 Screening

The two main screening tests used to detect PCa include the PSA test and a digital rectal exam (DRE). The PSA test consists of a blood test to measure a man's serum PSA levels and help assess his risk of PCa. Specifically, PSA is a component of seminal fluid that is secreted by epithelial cells within the prostate gland. Normally, very little PSA is found in the blood; however, increased PSA production, prostate growth, and tissue damage can all lead to increased levels of circulating PSA. Therefore, measuring the amount of PSA in the blood can be a useful indicator of abnormalities within the prostate gland; whilst this may be indicative of PCa, it may also reflect other non-malignant conditions, such as benign prostatic hyperplasia or prostatitis. Hence, controversies exist concerning the reliability of PSA testing to screen for PCa, as well as the potential overdiagnosis and overtreatment

that can result from testing. Nonetheless, the PSA test is still often used as a tool to help select those men who should have a follow-up prostate biopsy.

Like the PSA test, the DRE is also used to detect abnormalities in the prostate. Usually, a DRE is performed to provide further information about the physical characteristics of the prostate gland, particularly if the PSA result is inconclusive. This procedure involves an internal examination of the rectum to check for the presence of any irregular features (e.g. nodules, asymmetry, or induration) as well as the size of the prostate gland. However, controversies surround the ability of the DRE to accurately detect PCa and whether this procedure, like the PSA test, may result in the overdiagnosis and overtreatment of PCa instead.

1.1.3 Diagnosis

If cancer is suspected, then a needle biopsy of the prostate is typically performed to determine (i) whether cancer is present and, if so, (ii) the stage of disease. During this procedure, transrectal ultrasound (TRUS) imaging is used to help detect any abnormalities within the gland and identify tissue samples for biopsy. Following the biopsy, tissue samples are examined to assess whether cancer is present and the histological appearance of any cancer cells that are found. Specifically, cells are assigned a grade using the Gleason grading system based upon their level of differentiation from low to high (1-5) (Gleason, 1992). Within a tumour specimen, the two most common grades are then summed together to provide a Gleason score (2-10), aimed to reflect the aggressiveness of the disease (Gleason, 1992). Together with the results obtained from the DRE and the PSA test, the Gleason score is then used to stage the cancer.

PCa staging is the process of determining the extent and spread of disease. It is important in providing prognostic information and informing treatment decisions. The most commonly used staging system is known as the TNM system, which was developed by the Union for

International Cancer Control (UICC) to describe the size of the primary tumour (T), the involvement of regional lymph nodes (N), and the presence of distant metastasis (M). Two types of staging - clinical and pathological - are used in PCa. Clinical staging is based upon findings obtained about the tumour before surgery (e.g. the DRE result, PSA level, Gleason score and imaging tests), whereas pathologic staging is based upon the information gained by the gross and histological examination of the tumour following a radical prostatectomy or biopsy. By combining the clinical data with the (TMN) classifications, a stage of I, II, III or IV is then assigned to the disease, with stage I the least advanced and stage IV the most (Figure 1.1)

1.1.4 Management

The management of PCa varies depending on the stage and aggressiveness of the disease. Other factors that are considered include; the age of the patient at diagnosis, their health status, and personal preference. Overall, the treatment options available to patients with PCa are extensive and include both radical (i.e. prostatectomy, radiotherapy, androgen-deprivation therapy, chemotherapy) and conservative (i.e. active surveillance, watchful waiting) approaches. These treatments, however, can vary in both their effectiveness and side effects.

1.1.4.1 Radical approaches

Radical prostatectomy (RP) is a surgical (retropubic, laparoscopic or robotic) procedure that involves the removal of the prostate gland and seminal vesicles, where the ultimate goal is disease eradication. Reported side effects of RP include erectile dysfunction, infertility, and incontinence (Steinsvik et al., 2008; Hoyland et al., 2014; Emanu et al., 2016). Approximately 20-40 % of men who opt for radical prostatectomy exhibit biochemical recurrence (BCR) of the disease (i.e. increasing PSA levels); therefore, those patients who undergo RP are typically committed to at least 10 years of follow-up evaluations to facilitate

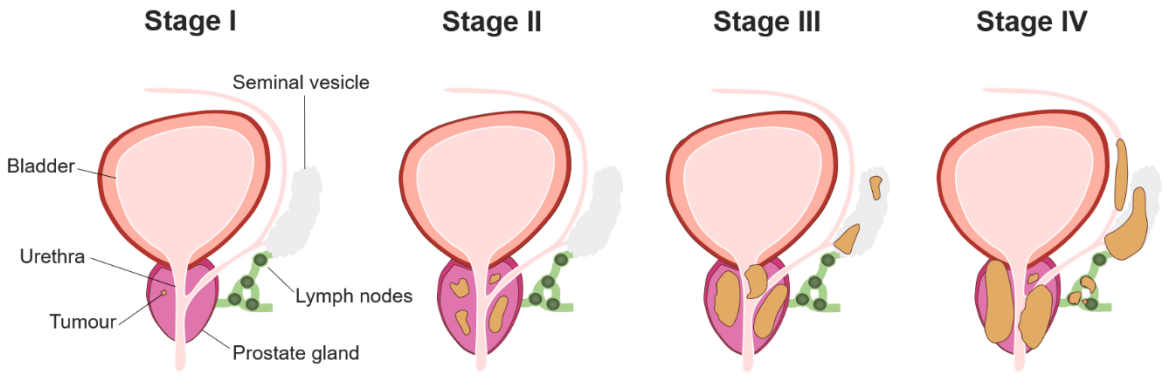


Figure 1.1. Stages of PCa. (I) The tumour is small and confined to the prostate. (II) The cancer is larger, but still confined to the prostate. (III) The cancer has spread beyond the prostate, but not to local or distal organs. (IV) The cancer has spread to nearby tissue such as the bladder, rectum and lymph nodes, and possibly to distal organs such as bone.

early intervention upon cancer recurrence (Amling et al., 2000; Freedland et al., 2005; Roehl et al., 2004).

Radiotherapy (RT) is a localised treatment that involves applying sources of ionizing radiation to tumour cells, typically with curative intent. Two types of radiation therapy - external beam radiation therapy (EBRT) and brachytherapy - are used to treat PCa, sometimes in combination. In EBRT, the source of radiation is external and directed towards the pelvis, whereas in brachytherapy, the radioactive source (low-dose or high-dose) is placed directly into the prostate gland. In both cases, radiation is used to damage PCa cells and prevent tumour growth. Side effects reported for RT are similar to those for RP (Resnick et al., 2013), but the rate of BCR differs between the two approaches depending on the patient risk group (Kim et al, 2015).

Androgen deprivation therapy (ADT) is a therapeutic approach used to decrease a man's levels of circulating testosterone – an androgen steroid hormone utilised by PCa cells to drive tumour growth. Testosterone levels can be reduced via the use of medication or surgery to inhibit the production of testosterone directly and/or to block its action in cells

and tissues. ADT can be used as part of treatment strategy with curative intent; for example, alongside RP or RT to improve the treatment effectiveness; or as a palliative option to manage disease symptoms. In terms of side effects, ADT is associated with several serious complications, ranging from cardiovascular and metabolic abnormalities, to reduced bone density and cognitive disturbances (Amirali et al., 2016).

Chemotherapy refers to the use of anti-cancer agents as a systemic therapy to prevent the proliferation of rapidly dividing cells. In PCa, chemotherapeutic drugs are typically administered to improve a patient's quality of life and palliate symptoms, although improvements in overall survival have also been reported when certain chemotherapy drugs are used in combination with other therapeutic agents (Tannock et al., 2004). Whilst chemotherapy is associated with several adverse effects, these are mostly mild in PCa, and chemotherapeutic drugs are generally well tolerated (Gravis et al., 2003).

1.1.4.2 Conservative approaches

Active surveillance is a treatment strategy aimed at managing PCa without surgical or therapeutic intervention until symptoms develop or the disease progresses. In this approach, patients are monitored over time for evidence of tumour growth or metastasis, with the overall goal being to postpone treatment for as long as possible and diminish patient morbidity. Indeed, as many PCa tumours grow very slowly, active surveillance enables patients to avoid the potential side effects of radical treatments until curative intervention is essential. Nonetheless, patients who opt to undergo active surveillance face higher risks of the disease advancing before such treatments are sought. However, multiple studies have reported that patients managed via active surveillance exhibit a low rate of progression to metastatic disease or death from PCa (Garisto and Klotz, 2017).

Watchful waiting represents another treatment approach also aimed at deferring immediate treatment following PCa diagnosis to avoid overtreatment of indolent disease. Patients are

monitored to assess PCa progression and treatments are administered if the disease advances. However, unlike active surveillance, in this strategy treatments are typically sought to manage disease symptoms rather than for curative purposes. Generally, watchful waiting is a treatment option limited to those patients deemed unfit to cope with the effects of radical treatment, or if the effects of treatment are expected to be more harmful than the cancer itself. Whilst immediate treatment following PCa diagnosis reportedly reduces long-term mortality more than watchful waiting (Bill-Axelson et al., 2014), patients also experience fewer adverse quality of life events (Kasperzyk et al., 2011).

1.1.4.3 PCa stage and treatment strategy

Localised PCa (Stage I and II) is cancer that is confined within the prostate gland and has not, therefore, spread to regional lymph nodes or metastasised to distant sites. Patients with localised PCa are stratified into groups according to their risk of recurrence and overall survival; this information is then used to direct treatment strategies. The treatment typically offered to men with intermediate-to high-risk disease is RP; while those with low-risk disease are managed with either radical or conservative treatments. At this stage, the goal of treatment is disease eradication; however, it is still unclear what the best option is to achieve this. The recent ProtecT trial set out to answer this question by comparing the long-term impacts of RP, RT, and active surveillance on PCa mortality and clinical outcomes in patients with localised PCa. After a median period of 10 years, the study reported that PCa–specific mortality was low (~1%) regardless of the treatment, although the impact on patient quality of life differed between the arms, and active surveillance was associated with a higher incidence of disease progression (Hamdy et al., 2016). Hence, a primary challenge in managing localised PCa involves balancing the survival benefits of treatment with the patient's potentially diminished quality of life.

Locally-advanced PCa (Stage III) is cancer that has spread outside of the prostatic capsule to areas such as the seminal vesicles, but not to regional lymph nodes or distant metastatic

sites. The population of patients diagnosed with locally advanced PCa includes a heterogenous group of men, some of whom may benefit from radical treatments and others who can be treated only palliatively. To optimally manage these patients, clinical and pathological staging tools are used to identify those at high risk of tumour recurrence after receiving local therapy. Currently, the treatment options available for high-risk PCa patients include RT, RP and ADT, either alone or in combination with local therapies. Most curative procedures used at this stage are based upon multidisciplinary strategies, combining radical therapies with neoadjuvant and/or adjuvant hormone therapy. However, given that more than 50% of high-risk men experience tumour recurrence after local therapy (Khan and Partin, 2002), the most effective treatment combination, as well as the timeframe in which hormone therapy should be instigated (i.e. before or after local therapy), is heavily debated. This remains one of the major challenges in managing locally advanced PCa.

Advanced PCa (Stage IV) is cancer that has metastasised outside of the prostate gland to other local and/or distal sites. At this stage, the disease is incurable and treatment approaches are used palliatively to manage symptoms, although tumour progression can be slowed for several years. Tumour heterogeneity is common in advanced PCa (Robinson et al., 2015) and known to influence treatment response. Although significant advances have been made, we currently lack the predictive tools to distinguish between such individuals – an issue which negatively affects clinical decision-making. Therefore, the treatment option frequently offered to most patients diagnosed with advanced PCa is ADT, alone or in combination with chemotherapy. Enrolment in a clinical trial or treatment with a novel or experimental therapy may also be recommended. Ultimately, although a multitude of hormonal agents and chemotherapeutics exist, all current treatments provide relatively modest survival benefits at this stage, yet numerous side effects. Therefore, the lack of effective and tolerable treatment options for advanced or recurrent PCa is one of the primary challenges in managing the disease at this stage.

1.1.5 Prognosis

In terms of prognosis, the 5-year survival rate for men with PCa in the UK is approximately 85% (Cancer Research UK, 2016), considerably higher than that of lung cancer (~8%), the second most common cancer in men in the UK. However, PCa prognosis varies considerably depending on the stage at which the disease is detected. In the UK, at the time of diagnosis, the five-year survival rate by stage ranges from approximately 100% at stages I – III, to ~30% at Stage IV, although this figure drops even further (<20%) for men diagnosed below the age of 50 (Cancer Research UK, 2016). Hence, advanced PCa is responsible for most PCa-related deaths. Therefore, developing therapeutic strategies to combat late-stage PCa represents a significant clinical aim.

1.2 Challenges in treating advanced PCa

As discussed, patients diagnosed with advanced PCa typically have poor prognosis. Although current treatment strategies may be able to delay disease progression and relieve symptoms for some time, they are not curative. Of the options available, the mainstay treatment approach for late-stage PCa is ADT; that is, to deplete the growing tumour of a sufficient androgen supply. Considering androgens play a critical role in the normal and pathogenic growth of the prostate (Lonergan and Tindall, 2011), one may then ask: how do PCa tumours progress to a lethal status if androgen signalling is blocked? To answer this question, closer examination of the role of androgens in the prostate and the androgen receptor (AR) signalling pathway is required.

1.2.1 The role of androgens in the prostate

In men, androgen steroid hormones, such as testosterone and dihydrotestosterone (DHT), are important for the development of both secondary sex characteristics and the sex-accessory glands, particularly the prostate (MacLean et al., 1993). Testosterone is the main circulating androgen in men and is produced mainly by the testes and to a lesser extent,

the adrenal glands. In the prostate, as well as in a number of other tissues, testosterone is converted to its more biologically active form, DHT, by the enzyme 5α-reductase. DHT is the primary androgen in the prostate and plays a pivotal role in gland development and growth (Tenniswood et al., 1982). For example, during embryogenesis, DHT mediates sexual differentiation of the prostate (Ablin and Farnsworth., 1989), whereas during puberty, DHT drives prostatic cell growth to complete the normal growth phase of the gland (Lee et al., 1995; Xue et al., 2001). Conversely, in the adult male, the role of DHT is seemingly reversed, and instead functions to maintain prostatic differentiation and suppress epithelial cell proliferation (Donjacour et al., 1993; Wu et al., 2007; Simanainen et al., 2007).

1.2.2 The AR signalling pathway

In order to regulate biological processes such as cell growth and differentiation in the prostate, DHT must signal via the AR - a ligand-dependent transcription factor and member of the nuclear receptor superfamily. Broadly, AR-mediated signalling involves an extracellular androgen signal stimulating a change in the transcriptional response of the cell (Figure 1.2). Specifically, in the absence of ligand (i.e. DHT or testosterone), the AR is localised to the cytoplasm, associated with a complex of various heat shock proteins and other chaperones. Upon ligand binding, a conformational change is induced in the AR which leads to dissociation of the chaperone proteins and AR dimerization. Together, the ligand-homodimer complex then translocates to the nucleus where it can bind to specific recognition sequences in the promoter and enhancer regions of target genes, known as androgen response elements (AREs). The recruitment of specific co-regulator proteins then completes the AR transcriptional complex and enables the AR to function as a positive or negative regulator of multiple cell cycle and differentiation genes.

1.2.3 AR signalling in advanced PCa

In the normal prostate epithelium, the AR acts to supress growth and maintain prostate differentiation. However, these functions seem to be overcome during the development of

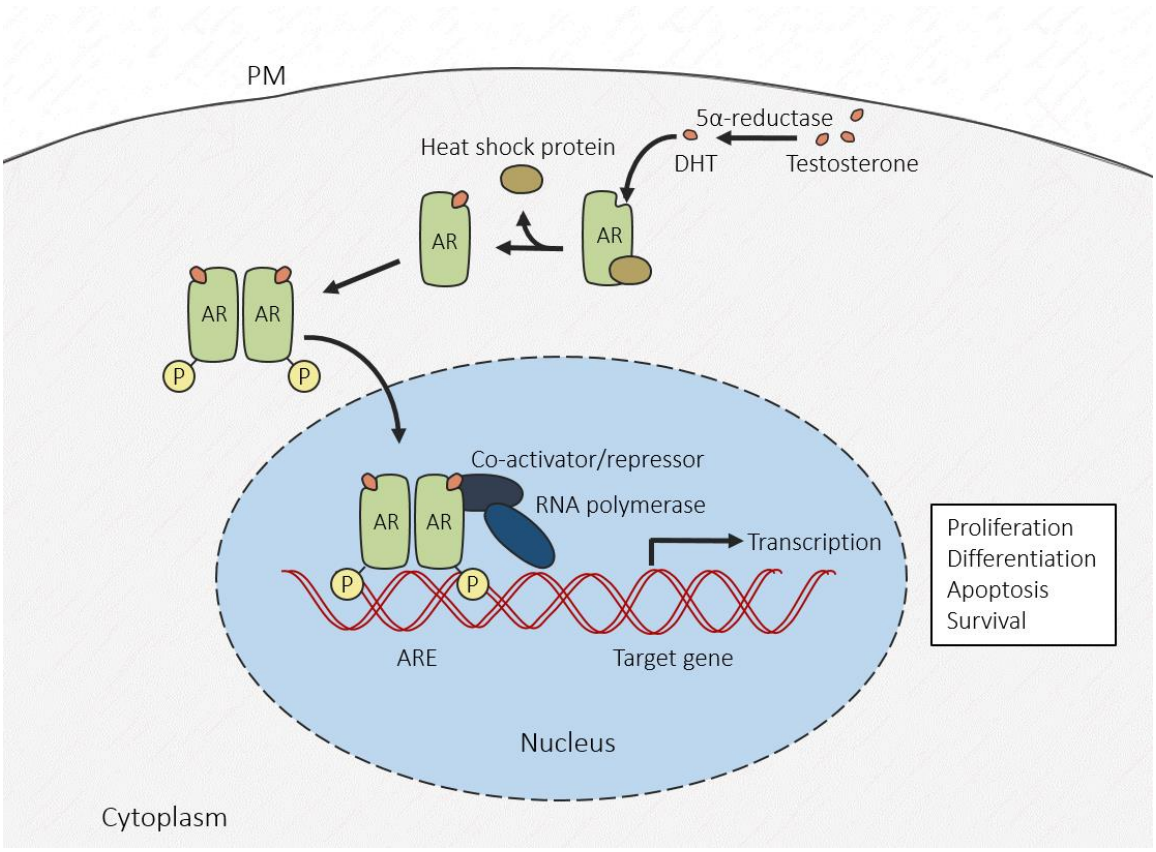


Figure 1.2. Overview of the AR signalling pathway. Testosterone enters the prostate epithelial cell and is converted into DHT by 5-alpha-reductase in the cytoplasm. Binding of DHT results in the dissociation of heat shock proteins, AR phosphorylation, dimerization and the translocation of the receptor to the nucleus. In the nucleus, the AR binds to the (ARE) of target genes, recruits coactivators/repressors, DNA transcriptional machinery, and regulates target gene expression.

PCa, where this pathway plays a key role in driving tumour growth instead (Gao et al., 2015). The dependency of PCa on AR activation for growth and survival was first established in the 1940's by Huggins and Hodges (Huggins and Hodges, 2002), who later demonstrated that blocking androgen production via castration could lead to significant improvements in the clinical condition of patients with advanced PCa (Huggins, Stevens and Hodges, 1941). Since then, androgen ablation (surgical or pharmacological) has been a mainstay in the treatment of advanced PCa and remains, to this day, one of the most effective therapies (Figure 1.3; Ferraldeschi et al., 2015). However, although the initial

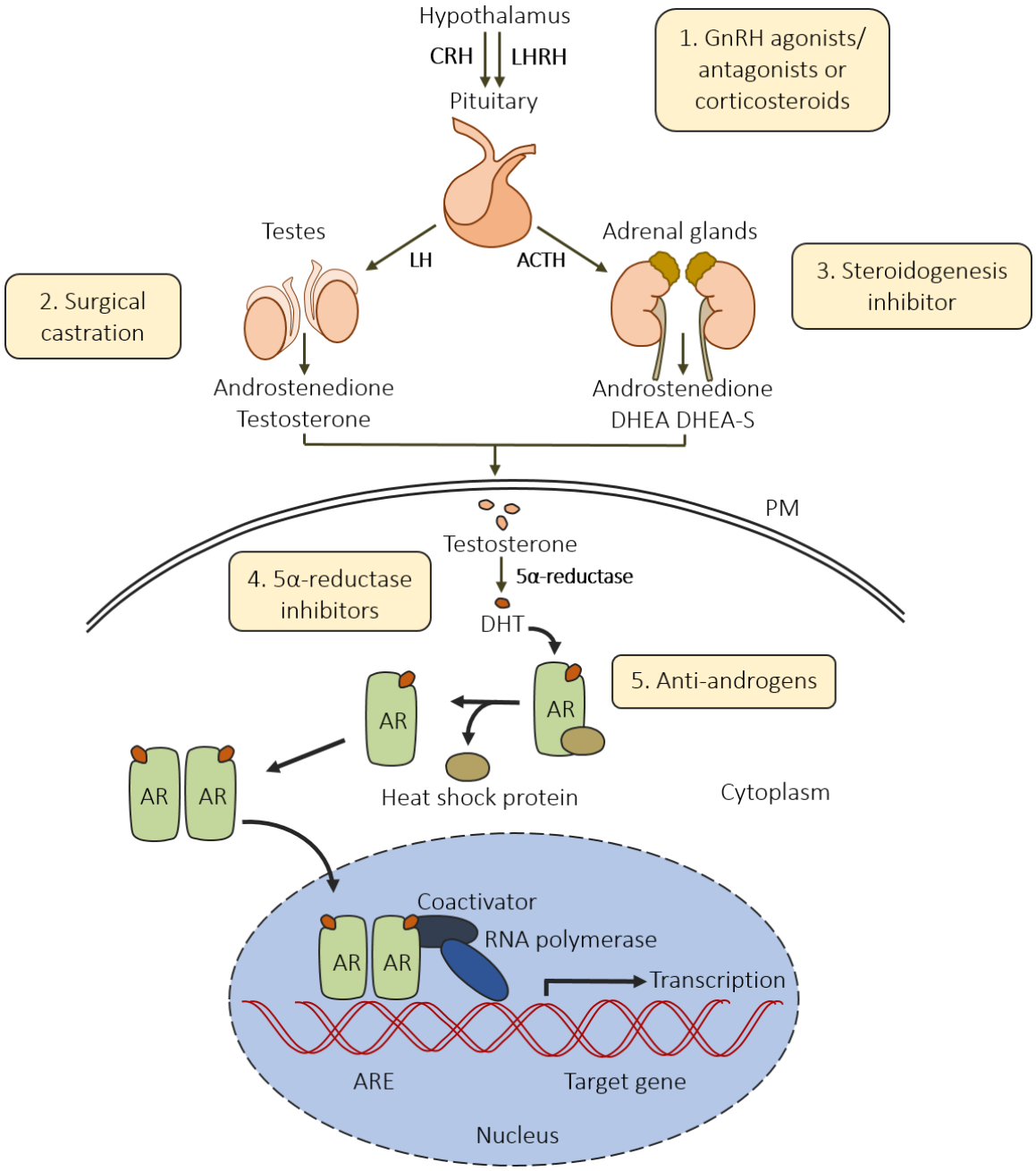


Figure 1.3. Targets of androgen ablation in PCa. In men, testosterone is mainly produced in the testes, but also in the adrenal glands as the precursors androsteinedione and dehydroepiandrosterone (DHEA). Luteinizing hormone (LH) and adrenocorticotropic hormone (ACTH) are secreted from the anterior pituitary gland and regulate testosterone production in the testes and adrenal glands, respectively. Upstream, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH) to control the level of these hormone. Androgen production can be inhibited in a number of ways: 1) Blocking LH secretion using GnRH agonists and antagonists, or ACTH secretion using corticosteroids. 2) Surgical castration, adrenectomy or hypophysectomy. 3) Inhibiting the activity of key enzymes in the androgen synthesis pathway. 4) Blocking the conversion of circulating testosterone to DHT by 5α-reductase via small molecule inhibitors. 5) Inhibiting the binding of agonists to the AR using competitive AR antagonists.

response rate can be up to 80%, patients inevitably relapse after a median period of 18 months (Crawford et al., 1989; Schellhammer et al., 1996; Caubet et al., 1997), having developed a more aggressive form of PCa that can grow despite diminished androgen levels, termed castration-resistant prostate cancer (CRPC). Historically, many believed that the CRPC pathology arose via AR-independent mechanisms. It is now understood however, that signalling through the AR continues to be crucial for tumour growth in a large proportion of CRPC patients.

1.2.4 Mechanisms underlying CRPC

Understanding how the AR signalling pathway functions under androgen-depleted conditions to drive tumour growth and castration-resistant disease has been a topic of intense research. A number of mechanisms have been implicated in the reactivation of this signalling axis following ADT; the majority of which are mediated by the AR and can be classified as either androgen-dependent or –independent (Figure 1.4).

1.2.4.1 Androgen-dependent mechanisms

Amplification of the AR gene is the most frequent genetic change observed among CRPC patients (Aggarwal et al., 2015). Enhanced AR expression via mechanisms independent of gene amplification, involving the activation of oncogenic signalling pathways, have also been linked to CRPC (Grad et al., 1999; Gregory et al., 2001; Sharma et al., 2010; Lin et al., 2013). Ultimately, high levels of AR are able to sensitise cells to the low levels of androgens that remain following ADT (Visakorpi et al., 1995; Waltering et al., 2009), and drive tumour growth despite castration.

Mutations in the AR gene is another common genetic change observed in CRPC (Culig et al., 1993; Taplin et al., 1995; Wallen et al., 1999). Indeed, more than one hundred somatic mutations have been identified in the AR (Taylor et al., 2010; Gottlieb et al., 2012), the

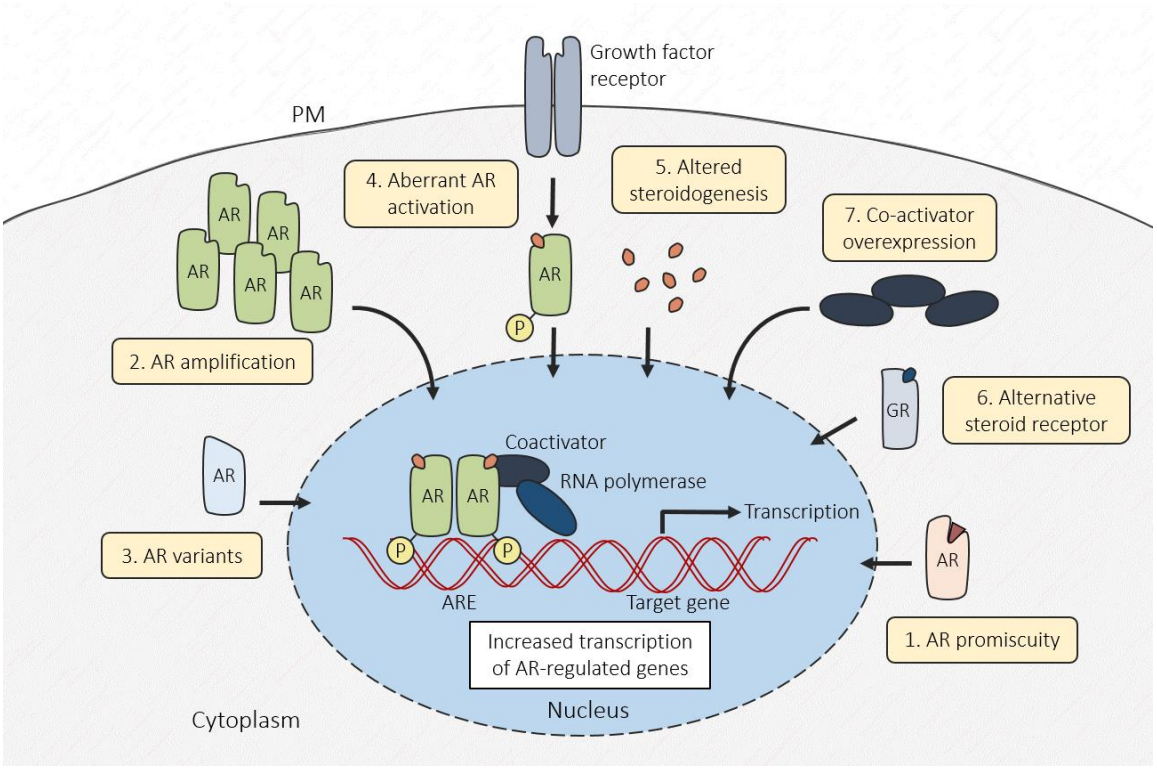


Figure 1.4. Mechanisms of CRPC. Continued transcriptional activity of the AR is a key driver of tumour progression in many cases of CRPC. Several mechanisms have been proposed to explain how PCa cells can maintain aberrant AR activity despite castrate levels of androgens. 1) Mutations in the AR can enable receptor activation by non-androgenic steroidal ligands or the conversion of AR antagonists into agonists. 2) Amplification of the AR gene or overexpression of the AR protein can promote receptor hypersensitivity to low androgen levels. 3) Splice variants of the AR, for example variants with truncated LBDs, can produce constitutive AR activation regardless of androgen levels. 4) Transactivation of the AR through growth factor signalling can stimulate AR transcriptional activity in the absence of androgens. 5) Increased adrenal and/or intra-tumoral androgen biosynthesis can also sustain AR transcriptional activity. 6) Expression of alternative steroid receptors with high sequence homology to the AR may be able to regulate a similar transcriptional program. 7) Overexpression of AR co-activators can enhance AR transcriptional activity in both an androgen-dependent and -independent manner.

majority of which are located in the N-terminal domain (NTD) and ligand-binding domain (LBD) of the receptor. As such, in low levels of androgens, mutations in the LBD can promote AR signalling by expanding the type of activating ligands to include non-androgenic steroids, such as progesterone and oestrogen (Steketee et al., 2002). Conversely, mutations in the NTD can promote constitutive AR activation by enhancing the transactivation of the receptor (Hay and Mcewan, 2012).

Altered expression of AR co-regulators is another mechanism associated with CRPC development. Using this strategy, the cell can modulate the interactions of the AR with its co-regulators and enhance receptor transactivity even in low androgen conditions (Hermanson et al., 2002; Chmelar et al., 2007; Jozwik et al., 2012). Generally, increased expression of co-activators enhances the constitutive activity of the AR (Wolf et al, 2008), whereas the opposite is observed for co-repressors (Huang et al., 2018). As such, co-activator expression is frequently upregulated in castrate-resistant tumours (Xu et al., 2009), whereas co-repressor expression or recruitment is typically reduced (Godoy et al., 2012).

Altered steroidogenesis to restore intra-tumoral androgen levels has been proposed as another strategy employed by cells to sustain tumour growth following ADT (Geller et al., 1978; Mostaghel et al., 2007). High levels of androgens persist in CRPC tumours despite androgen ablation (Montgomery et al., 2008), and are thought to be generated from androgen precursors derived from the adrenal glands (Titus et al., 2005; Sharifi et al., 2013; Chang et al., 2014), or synthesised *de novo* within tumour tissues (Locke et al., 2008; Knuuttila et al., 2014). Increased expression of steroidogenic enzymes is also commonly observed in CRPC tumours (Titus et al., 2005; Stanbrough et al., 2006; Yepuru et al., 2013).

1.2.4.2 Androgen-independent mechanisms

Expression of AR splice variants (AR-Vs) in advanced PCa is associated with resistance to ADT (Nakazawa et al., 2014). By expressing AR-Vs, cells may be able to maintain AR transactivation without the normal regulatory controls. Indeed, most AR-Vs lack the LBD found in full-length AR and can, therefore, remain constitutively active even in androgen-depleted conditions (Dehm et al., 2011; Haile et al., 2011). One such variant, AR-V7, is frequently overexpressed in CRPC (Guo et al., 2009; Hu et al., 2012), and increased levels of AR-V7 are associated with hormone-refractory disease progression (Qu et al., 2015).

Expression of alternative steroid receptors has been proposed as a mechanism to promote CRPC tumour growth via both androgen- and AR-independent routes. Steroid receptors such as the glucocorticoid receptor (GR) and progesterone receptor (PGR) share high sequence homology with the AR (Chen et al., 1997) and may, therefore, be able to regulate a similar transcriptional programme (Sahu et al., 2013). Expression of the GR is increased in patients following ADT (Szmulewitz et al., 2012), and high levels of the GR are associated with disease progression (Grindstad et al., 2015); hence, redundancy between the AR and these receptors may promote CRPC in certain patients.

Aberrant AR activation by different signalling pathways is also linked to CRPC development. The tumour microenvironment provides various mitogenic signals, such as growth factors and cytokines, that can activate signal transduction cascades to amplify AR activity (Culig et al., 1994; Wen et al., 2000; Ueda et al., 2002) and drive PCa progression (Schayowitz et al., 2008; Traish and Morgentaler, 2009). Several preclinical studies have demonstrated that, downstream of these signals, intracellular kinases such as Src (Guo et al., 2006), Ack1 (Mahajan et al., 2007), NF-κB (Nadiminty et al., 2013) and Akt (Xin et al., 2006) can enhance the transcriptional activity of the AR by phosphorylating the receptor directly and/or certain co-regulators (Lamont and Tindall, 2011). In this way, signalling pathways that are activated in advanced PCa may be able to augment tumour growth despite castrate-levels of androgens.

1.2.4.3 Future perspectives

Overall, current hormone therapies which target the androgen signalling axis are often effective in delaying disease progression and managing symptoms in patients with advanced PCa. However, given that all patients inevitably develop incurable castration-resistant disease, new therapeutic strategies are urgently needed. Non-hormonal therapies which target the crosstalk between growth factor and AR signalling pathways holds significant clinical potential. Normal androgen-dependent prostate growth is regulated by

paracrine growth factor signalling (Cunha & Donjacour, 1989; Byrne *et al.,*1996), and the interplay between these pathways is thought to underpin the development of androgen-independent growth in certain patients (Zhu and Kyprianou, 2008). Therefore, inhibition of the AR and growth factor signalling pathways, together, may prove a more effective therapeutic strategy to prevent CRPC transformation and evolution. However, further work is needed to uncover how the growth factor pathways that are active in advanced PCa may contribute to tumour growth.

1.3 The tumour microenvironment (TME)

In recent years, our understanding of tumorigenesis has changed. No longer do we consider tumours to be masses of epithelial cells undergoing uncontrolled cell proliferation in isolation; now, we recognise that tumorigenesis is a dynamic process dependent upon a complex set of interactions between cancer epithelial cells and the surrounding microenvironment. Within the TME, non-malignant cells and their associated stroma undergo genetic alterations following tumour-stroma interactions to promote the malignant phenotype (Dudley et al., 2008; Hill et al., 2005; Krušlin et al., 2015), and disease progression (Tuxhorn et al., 2001; Karlou et al., 2010). Underlying this intercellular communication is a host of secreted growth factors, chemokines, and cytokines, as well as an array of molecular machinery required to transduce these signals in recipient cells (Josson et al., 2010). Dissecting the mechanisms by which these factors govern the malignant behaviour of cancer is therefore necessary if we are to exploit the TME for therapeutic gain.

1.3.1 The TME in advanced PCa

In advanced PCa, the TME in which secondary tumours are formed typically concerns that within bone marrow (BM), given that approximately 90% of patients with late-stage PCa exhibit metastatic lesions here (Bubendorf et al., 2000). Such figures are believed to reflect,

in part, the permissive environment offered by the bone marrow to disseminated PCa tumour cells. Abundant in progenitor cells with the capacity to differentiate into numerous auxiliary cell types, such as endothelial cells, myofibroblasts and fibroblasts, the BM is rich in the resources required to produce a functional stroma and support PCa progression. In order to become established and thrive in this environment, however, PCa cells must interact and co-evolve with the stroma to develop a specialised phenotype. This can be a timely process and is one possible reason why tumour cells may remain dormant in these secondary sites for some time. Indeed, most patients with localised PCa who develop bone metastases do so several years after primary tumour resection (Morgan et al., 2009). As the disease advances, certain signals are believed to waken tumour cells. No longer dormant and proliferative in phenotype, these cells then drive metastatic outgrowth. Following hormone therapy, additional signals within this environment have been implicated in driving castrate-resistant tumour growth (Lee et al., 2014). The growth of PCa cells in the BM has numerous consequences, two important ones include promoting the formation of new bone and interrupting normal bone homeostasis, both of which highlight why this event is fatal in patients. Hence, understanding how PCa cell growth is regulated by factors present in the bone microenvironment could have important clinical implications.

1.3.2 Extracellular signals in the TME: BMPs

One set of factors enriched within the bone matrix (Wozney et al., 2002; Blum et al., 2004) and expressed by PCa cells (Harris et al., 1994) are the bone morphogenetic proteins (BMPs). BMPs comprise a class of more than 20 extracellular signalling molecules that constitute the largest branch of the transforming growth factor-ß (TGF-ß) superfamily (Rahman et al., 2015). Although BMPs were originally identified by their ability to induce ectopic bone formation, it has become evident that these cytokines have broader functions and can regulate biological processes as diverse as cell proliferation, differentiation and cell fate determination (Hogan, 1996; Chen et al., 2004). Considering the well-described osteogenic properties of BMPs (Reddi, 1998), many studies completed so far have focused

on the involvement of these ligands in the production of osteoblastic lesions in bone-metastasised PCa (Dai et al., 2005; Feeley et al., 2005; Ibrahim et al., 2010). However, there is also interest in understanding the role BMPs may play in regulating cell growth in advanced PCa, especially given that several members of the TGF-ß family are known to regulate this event in other processes such as embryogenesis and organogenesis (Lyons et al., 1989; Jones et al., 1991).

1.3.2.1 The BMP signal transduction pathway

To regulate processes such as cell growth, receptor binding by extracellular BMP at the plasma membrane must be transformed into an intracellular response (Figure 1.5). In the canonical signalling pathway, BMPs initiate a Smad-mediated signal transduction cascade that culminates in altered transcription of select target genes. Specifically, BMP ligands bind to their cognate receptors, forming a ligand-receptor complex that consists of two dimers of Type I and Type II serine/threonine kinase transmembrane receptors and one BMP ligand dimer (Miyazono et al., 2010). Mechanistically, the constitutively active Type I receptor then transphosphorylates and, therefore, activates the Type II receptor. In turn, the activated Type I receptor phosphorylates the downstream effector proteins known as receptor-regulated Smads (R-Smads). R-Smads involved in BMP signalling (i.e. Smad1, Smad5, and Smad8) then complex with the co-mediator Smad (co-Smad), Smad4, translocate together into the nucleus and bind to DNA to regulate gene expression. Two important transcriptional targets of this pathway are the inhibitory-Smads (I-Smads), Smad6 and Smad7. These I-Smads compete with Smad4 for binding to R-Smads, antagonising the activity of the R-Smad/Co-Smad complex. Consequently, I-Smads mediate negative feedback of the pathway, downregulating BMP signalling in response to increased BMP activity. Hence, BMP signalling is normally tightly controlled, but alterations in the pathway can lead to aberrant signalling, as is frequently observed in PCa.

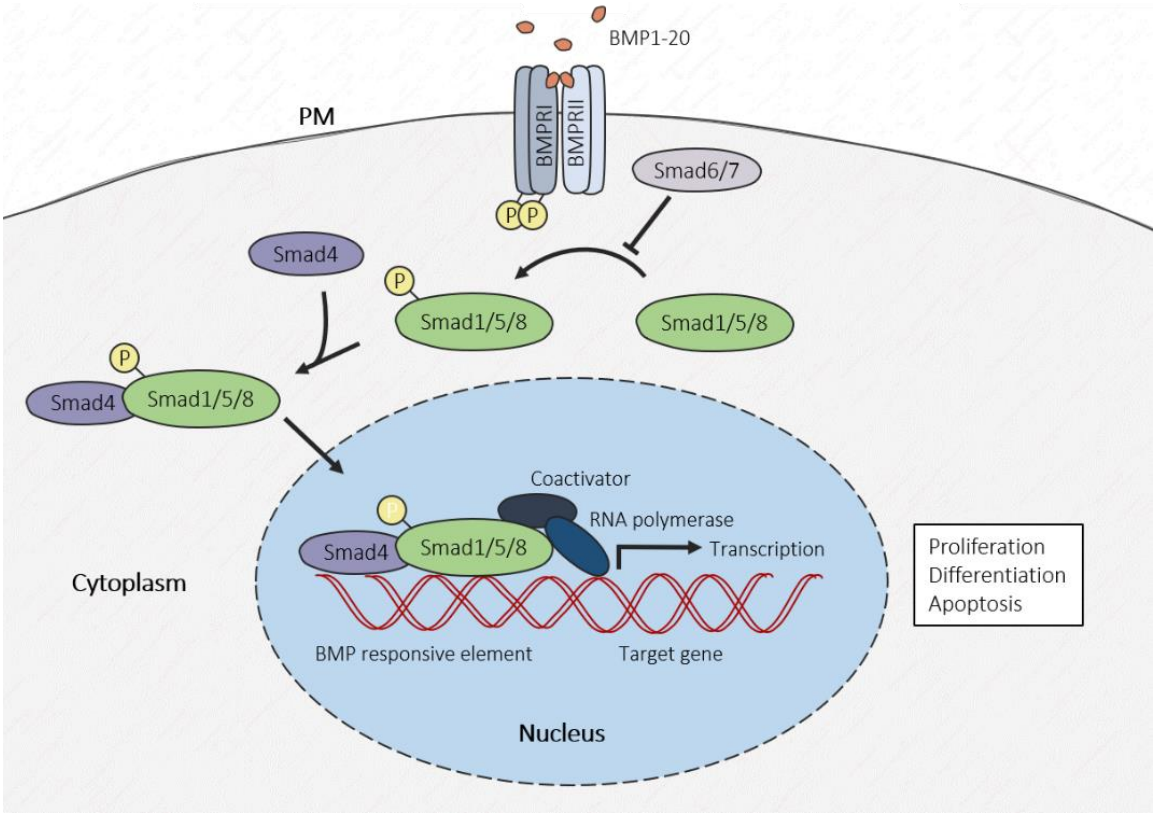


Figure 1.5. Overview of the canonical BMP signalling pathway. BMP ligands form homo- or heterodimers and bind a tetrameric set of cell-surface receptors, comprising two type I and two type II receptors. BMP binding catalyses a phosphorylation cascade that results in the phosphorylation of Smad1/5/8. Phosphorylated Smad1/5/8 complexes with Smad4 and then translocates to the nucleus to regulate gene expression via interactions with certain co-factors. Examples of cellular processes regulated by BMP signalling are given.

1.3.2.2 Alterations in BMP signalling in PCa

The expression of certain BMP signalling components are altered as PCa progresses, indicating that signalling via this pathway may be involved in tumorigenesis. For instance, reduced expression of certain BMP ligands, BMP receptors and SMAD proteins have been observed in tumour samples derived from patients with advanced PCa compared to those with localised disease (Kim, et al., 2000; Kim et al., 2004; Masuda, et al., 2004; Hovarth et al., 2004; Aitchison et al., 2008; Ding et al., 2011), hinting that decreased BMP signalling may promote PCa progression. Conversely, findings reported by our clinical collaborators (Hamdy et al., 1997; Autzen et al., 1998) have shown that increased expression of certain BMP ligands correlates with the development of advanced PCa, suggesting the opposite

conclusion. Furthermore, other studies have revealed that the expression patterns of some BMPs are even more complex and fluctuate across all stages of disease (Masuda et al., 2004; Thomas, et al., 2001). Therefore, the general role of BMP signalling in PCa progression remains unknown.

1.3.2.3 BMP signalling regulates PCa cell growth

Many have attempted to understand the complex expression data detailed above by examining the functional effects of aberrant BMP signalling through genetic manipulation studies. However, studies investigating the effects on cell growth have been equally confusing, as many have revealed that BMP signalling can promote both anti- and pro-proliferative responses in PCa cells. For instance, overexpression of BMP cell-surface receptors and downstream Smad effector proteins have each been shown to inhibit PCa cell proliferation *in vitro* (Qiu et al., 2007). Similar effects on PCa tumour growth have been observed *in vivo*, following overexpression of certain BMP ligands (Morrissey et al., 2010) or silencing of the BMP antagonist Noggin (Secondini et al., 2011). Alternatively, others have shown that PCa tumour growth can be enhanced *in vivo* by overexpression of a dominant-negative form of the BMP receptor BMPRII (Kim et al., 2004). Altogether, these findings provide strong evidence that BMP signalling serves to inhibit tumour growth in PCa.

However, equally convincing findings by others support a role for BMP signalling in promoting tumour growth and PCa progression. For example, using a murine model of PCa, one study demonstrated that by blocking BMP ligands from binding to their cognate receptors using anti-BMP antibodies, they could inhibit intraosseous tumour growth (Dai et al., 2005). Similar findings were reported by another group examining the effects of Noggin overexpression in murine PCa cell lines *in vitro* (Pham et al., 2013). Nonetheless, the same study using the same approach *in vivo*, showed that overexpression of Noggin elicited a growthy-inhibitory response in PCa cells. Others have reported similarly conflicting results when investigating the effects of recombinant BMPs on PCa cell growth

in culture; with some showing that BMP ligands can stimulate cell proliferation (Yang et al., 2006; Yang et al., 2005; Lee et al., 2014) and others demonstrating the opposite effect (Ide et al., 1997; Haudenschild et al., 2004; Miyazaki et al., 2004).

Overall, these seemingly contradictory findings have led to the conclusion that BMP signalling may exert distinct effects during different stages of PCa tumorigenesis. Indeed, signalling via this pathway is mediated by a large number of BMP ligands and BMP receptors, allowing for a diverse range of downstream processes to be affected by the same signalling cascade. Hence, altered expression of these components, as is observed in PCa, may enable BMP signalling to regulate different functions as the disease progresses. Therefore, individual signalling components need to be examined separately to correlate their function with the clinical expression data. An additional point to consider is that individual BMP components have been reported to elicit different effects in the same PCa cell lines, suggesting that the function of these signalling molecules may vary depending on the microenvironmental context. Therefore, studies using multiple PCa cell lines and a range of different culture conditions are necessary if we are to gain a more complete understanding of the ways in which BMP signalling affects tumour growth in PCa.

1.3.2.4 BMP signalling and CRPC

Although the effect of BMP signalling on PCa cell growth remains a controversial topic, a small number of studies have hinted that activation of this pathway in advanced PCa may promote the development of castrate-resistant disease in certain patients. For instance, BMP signalling has been shown to enhance PCa cell growth in androgen-deprived conditions *in vitro* (Lee et al., 2014) and *in vivo* (Lee et al., 2013). Specifically, one of these studies demonstrated using a co-culture system, that activation of BMP signalling in PCa cells was induced by the bone stroma and responsible for the stimulatory effects observed on growth (Lee et al., 2014). They further showed that the bone stroma could promote AR expression, consistent with findings reported by others that BMP signalling enhances AR

activity and androgen hypersensitivity in PCa cells (Lee et al., 2013; Yang et al., 2014). Hence, this work has led to the theory that in advanced PCa, the bone microenvironment promotes BMP signalling in PCa cells, increasing androgen-independent AR activity, cell growth, and ultimately, castrate-resistant disease.

Studies performed by our collaborators have hinted that a parallel regulatory mechanism may exist in *Drosophila*. Using the *Drosophila* accessory gland as a model for the human prostate, a previous PhD student showed that hyperactive BMP signalling in specialised secretory cells within this organ could enhance cell growth (Leiblich et al., 2012). Moreover, he revealed that the stimulatory effects of BMP signalling on cell growth were mediated by a nuclear hormone receptor known as the ecdysone receptor (EcR). Specifically, he showed that BMP signalling enhanced EcR levels via a post-transcriptional mechanism. As the EcR shares many characteristics with the AR (Wilson et al, 2017), it would be interesting to explore whether a similar level of regulation exists in human PCa cells. Indeed, such a finding would surely be important in highlighting novel clinical targets for therapeutic intervention during the advanced stages of PCa.

1.3.3 Extracellular signals in the TME: nutrients

In addition to growth factors (e.g. BMPs), the TME is rich with a host of other extracellular stimuli such as amino acids, glucose, and oxygen, which can promote or limit the growth of the developing tumour, depending on their relative abundance. Together, these nutrients and growth factors regulate processes such as cell growth and metabolism, in both physiological and pathophysiological conditions, via their actions on the downstream signalling hub known as the mechanistic target of rapamycin (mTOR).

1.3.3.1 The mTOR complexes

Target of rapamycin (TOR) is a highly conserved serine/threonine protein kinase and member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of proteins (Keith

and Schreiber, 1995). The TOR genes, TOR1 and TOR2, were originally identified through seminal studies conducted in yeast, as mutations in these genes were found to confer resistance to the macrolide rapamycin (Heitman et al., 1991; Kunz et al., 1993). Every eukaryote genome examined so far has been shown to contain a TOR gene, although typically only one (Heitman et al., 1991; Kunz et al., 1993). This is true in mammals, where mammalian (or mechanistic) TOR (mTOR) is known to be the sole target of rapamycin, encoded by a single mTOR gene (Sabatini et al., 1994).

mTOR nucleates two functionally distinct heteromeric complexes, mTORC1 and mTORC2 (Figure 1.6). Although these complexes were initially distinguished by their sensitivity to rapamycin, with mTORC1 and mTORC2 functions appearing rapamycin-sensitive and rapamycin-insensitive, respectively (Loewith et al., 2002; Sarbassov et al., 2004), we now know that rapamycin-resistant mTORC1 functions exist and that mTORC2 function can become rapamycin-sensitive following prolonged treatment (Sarbassov et al., 2006). In terms of their structure, in addition to mTOR, the two complexes contain several other evolutionarily conserved core subunits. For instance, mTORC1 contains regulatory-associated protein of mTOR (raptor) and proline-rich Akt substrate 40 kDa (PRAS40); whereas mTORC2 contains rapamycin-insensitive companion of mTOR (rictor), stress-activated protein kinase-interacting protein (Sin1) and protein observed with ricTOR (protor). Both also contain DEP Domain Containing mTOR-Interacting Protein (Deptor) and mammalian lethal with SEC13 protein 8 (mLST8) (Laplante and Sabatini, 2009).

1.3.3.2 The mTORC1 signalling pathway

The distinct structural composition of mTORC1 and mTORC2 allows for independent regulation and functioning. The mTORC1 signalling pathway, the main focus of my work here, primarily serves to coordinate processes such as cell growth and metabolism with environmental inputs to ensure optimal functioning of the cell. As such, mTORC1 is widely recognised as one of the key players involved in mediating the adaptive response of normal

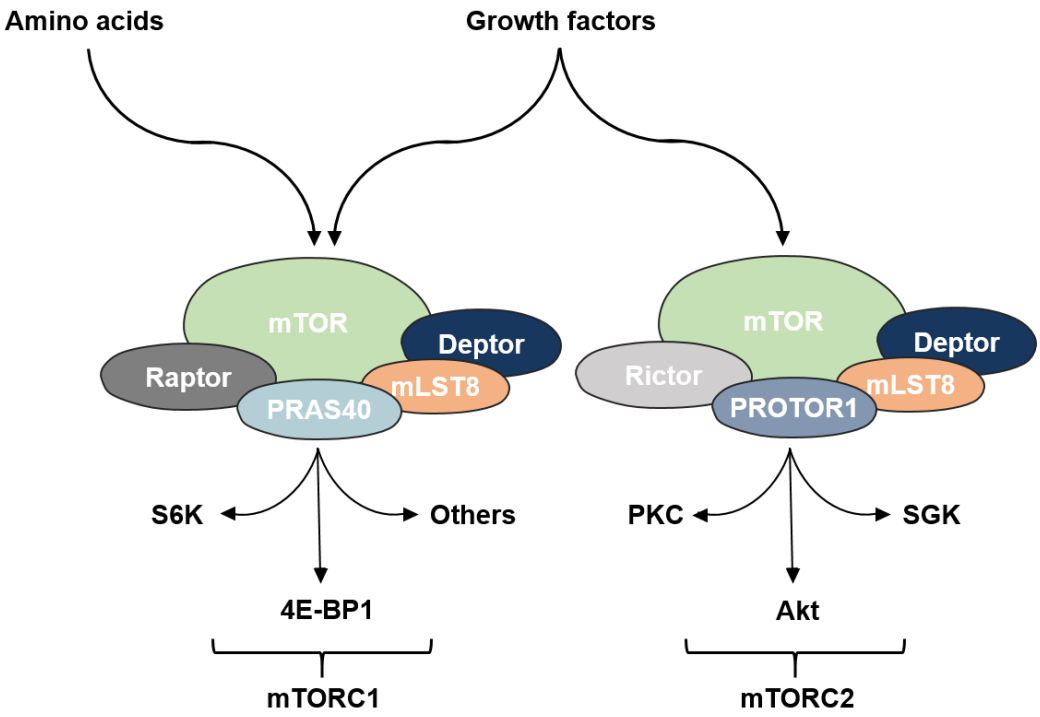


Figure 1.6. Components of mTORC1 and mTORC2. The most well-known substrates and upstream regulators of the two mTOR-containing complexes are shown.

and tumour cells to their surrounding microenvironment. Mechanistically, the way in which mTORC1 regulates these events is via phosphorylation of downstream effector proteins. One of the major functions of mTORC1 is to positively control protein synthesis, a necessary event to sustain cell growth. Indeed, two of the best characterised downstream targets of this pathway are the translational components eukaryotic initiation factor 4E binding protein-1 (4E-BP1) and the ribosomal protein S6 kinase (S6K). Phosphorylation of these targets by mTORC1 stimulates the initiation of protein synthesis. In addition, mTORC1 acts to positively regulate numerous other anabolic programs like the biosynthesis of lipids and organelles, as well as limit catabolic activities such as autophagy.

Conversely, our understanding of mTORC2 signalling is still in its infancy. Nonetheless, important roles in which mTORC2 has been implicated include cell survival, migration, metabolism and cytoskeleton organisation (Dada et al., 2008). Largely, the way in which mTORC2 regulates these processes is through phosphorylation of the substrates Akt,

protein kinase C α (PKCα) and serum/glucocorticoid-regulated kinase 1 (SGK1) (Sarbassov et al., 2005; Jacinto et al., 2008; Gan et al., 2012). Similar to mTORC1, mTORC2 is also controlled by growth factors (Guertin and Sabatini, 2007), however the mechanisms underpinning mTORC2 regulation remain incompletely understood.

1.3.3.3 Regulation of mTORC1 activity

Given its role in balancing the anabolic and catabolic interests of the cell, mTORC1 is itself controlled by a myriad of extracellular signals to ensure that signalling is active only in favourable conditions (Figure 1.7). One of the main ways in which these signals alter mTORC1 activity is by controlling the key negative regulator of this pathway, tuberous sclerosis complex (TSC). TSC is a heterodimeric complex comprised of TSC1 (hamartin), TSC2 (tuberin) and TBC1D7 and serves as a GTPase-activating protein (GAP) for the small GTPase Ras homolog enriched in brain (Rheb), a direct activator of mTORC1 (Inoki et al., 2003). As such, active TSC inactivates Rheb, and mTORC1 activity is inhibited in turn (Manning and Cantley, 2003). The activity of TSC is controlled by several kinases which serve to phosphorylate this protein, promoting either activation (e.g. Akt, ERK, S6K) or inactivation (e.g. AMPK and GSK3β) of mTORC1 (Manning et al., 2002; Huang and Manning, 2008; Inoki et al., 2003; DeYoung et al., 2008; Roux et al., 2004). Although TSC activity is primarily regulated by growth factors and mitogenic signals, more recent studies have also implicated a key role for amino acids. Nonetheless, the most well-characterised way in which amino acids signal to mTORC1 is via the Rag GTPases.

It has long been appreciated that amino acid levels are critical for mTORC1 activation and represent one of the most conserved growth signals to this pathway. A significant breakthrough which has fostered our understanding of how amino acids regulate mTORC1 activity was the identification of the Rag GTPases. The Rag family of proteins consist of four GTPases (Rag A, B, C and D) anchored onto the late endosome/lysosome (LEL)

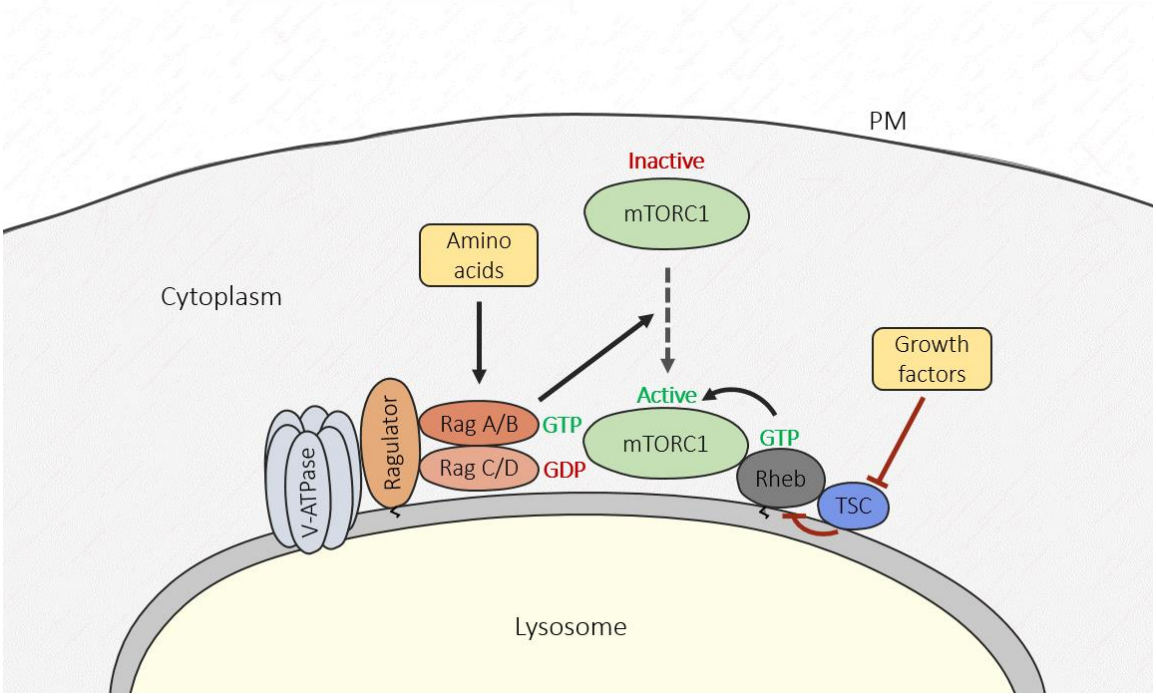


Figure 1.7. Overview of mTORC1 activation. Various stimuli can alter mTORC1 signalling, although the two well-characterised upstream regulators include amino acids and growth factors. Growth factor receptor binding leads to the phosphorylation and inactivation of TSC, a GAP for the GTPase and mTORC1 activator Rheb; both of which are localised to the lysosome. Simultaneously, amino acids enter the cell and promote the active form of the Rag-GTPases, RagA/B^{GTP}–RagC/D^{GDP}. Following this, RagA/B^{GTP}–RagC/D^{GDP} interact with mTORC1 and translocate the complex to the lysosome through interactions with the Ragulator. In this way, amino acids and growth factors are integrated to coordinate the simultaneous stimulation of the mTORC1 activator (Rheb) with the translocation of mTORC1 to its site of activation (the lysosome), ensuring both strict control and maximal activation of this pathway.

membrane via associations with a scaffolding complex known as the Ragulator, where they function as obligate heterodimers of Rag A or B together with Rag C or D (Bar-Peled et al., 2012). Amino acid stimulation drives the active nucleotide-bound Rag GTPase conformation, enabling Rags to interact with mTORC1 and recruit the complex to the LEL surface (Sancak et al., 2008). Localisation to the LEL membrane then allows mTORC1 to interact with Rheb, also located here, and become activated (Zoncu et al., 2011). In this way, amino acids and growth factors are integrated to coordinate the simultaneous stimulation of the mTORC1 activator (Rheb) with the translocation of mTORC1 to the site of activation (the LEL), ensuring both strict control and maximal activation of this pathway.

It is therefore unsurprising that alterations in these regulators are frequently observed in cancer, particularly PCa (Edlind and Hsieh, 2014).

1.3.3.4 Alterations in the mTORC1 pathway in PCa

Aberrations in the PI3K-AKT-mTORC1 pathway are extremely common in PCa and have been reported in 42% of patients with localised PCa and 100% of patients with advanced disease (Taylor et al., 2010). Such figures suggest that signalling via this pathway may be important in both PCa development and progression. Indeed, knockout and transgenic mouse models have revealed that hyperactivation of the PI3K-AKT-mTORC1 signalling axis is sufficient for the development of PCa *(*Majumder et al., 2003; Trotman et al., 2003; Wang et al., 2003), whereas inhibition of mTORC1 activity using pharmacological agents can reduce the growth of established PCa tumours *in vivo* (Kinkade et al., 2008; Nardella et al., 2009). Alterations in this pathway are also associated with androgen insensitivity and the development of CRPC (Wang et al., 2003; Jiao et al., 2007). Several studies have revealed that crosstalk between PI3K-AKT-mTORC1 and the AR signalling pathway can promote androgen-independent PCa cell growth (Carver et al., 2011; Mulholland et al., 2011), and that dual inhibition of these pathways can delay the development of CRPC in human PCa xenografts (Carver et al., 2011; Thomas et al., 2013).

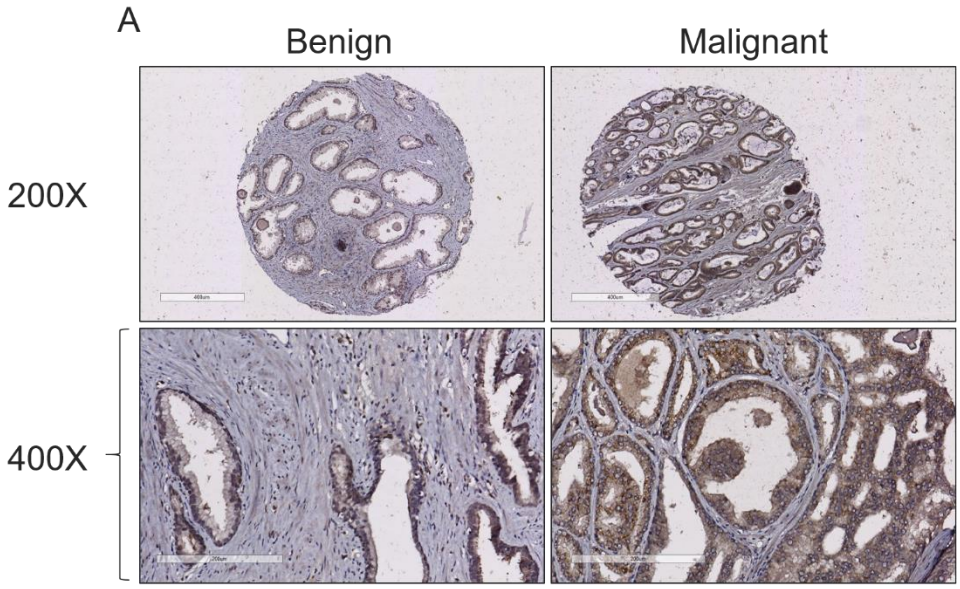
1.3.3.5 Targeting mTORC1 in PCa

However, despite encouraging findings in preclinical studies, mTOR inhibitors have shown limited efficacy in phase I-II clinical trials in PCa patients (Armstrong et al., 2010; Nakabayashi et al., 2012; Templeton et al., 2013). Proposed explanations for the poor clinical performance include the fact that first generation mTOR inhibitors only block partial mTOR function. Indeed, many of these agents are analogues of the allosteric mTORC1 inhibitor rapamycin (i.e. rapalogues) and, therefore, inhibit the phosphorylation of only a subset of substrates (Choo et al., 2008; Kang et al., 2013). The advent of second-generation inhibitors that directly target the mTOR catalytic site have since highlighted the

importance of blocking all mTOR kinase activity in PCa. For example, the second-generation inhibitor INK128 was shown to decrease tumour burden in a murine model of PCa to a significantly greater extent than the rapalogue everolimus (Hsieh et al., 2010). Given that kinase inhibitors of mTOR target both mTORC1 and mTORC2, it has largely been assumed that inhibition of the latter complex is responsible for the improved therapeutic response. However, mechanistic studies have revealed that inhibition of mTORC1 rapamycin-resistant substrates may be more important than inhibition of mTORC2 (Feldman et al., 2009; Thoreen et al., 2009; Hsieh et al., 2010), and that therapeutic agents which block the phosphorylation of these substrates might provide a more targeted response. Nonetheless, components of this pathway which specifically regulate rapamycin-resistant mTORC1 have yet to be identified in PCa.

Recent work conducted in our lab has shown that proton-assisted amino-acid transporter 4 (PAT4/SLC36A4) is a positive regulator of rapamycin-resistant mTORC1 signalling in colorectal cancer cells (Fan et al., 2016). Indeed, in HCT116 cells, amino acid stimulation has been shown to enhance rapamycin-resistant mTORC1 signalling in a PAT4-dependent manner; whereas, in HEK-293 cells, PAT4 overexpression can promote rapamycin-resistance. Interestingly, a DPhil student in the lab recently showed that PAT4 levels are upregulated in PCa (Dan Stevens, unpublished data). Using tissue microarray (TMA) analysis, he found that PAT4 protein was significantly increased in tumour samples derived from malignant prostate tissue versus benign tissue (Figure 1.8; Table 1.1). Moreover, other published reports have shown that changes in PAT4 copy number and mRNA expression are significantly more common in metastatic than localised PCa and are associated with both biochemical and metastatic recurrence following RP (Taylor et al., 2010). Hence, PAT4 may play a role in both PCa development and progression and could potentially serve as a novel therapeutic target in the treatment of rapamycin-resistant PCa. However, the role of PAT4 in PCa is currently unknown.

A



B

	Benign	Malignant
Cytoplasm IRS (mean)	2.41	6.54
Membrane (mean)	4.66	3.26
P value (two tailed)	<0.001	0.02
No. of samples	44	137

Figure 1.8. PAT4 protein expression in prostatic tissue. (A) Benign and malignant prostatic tissue was analysed via immunohistochemistry using an 'in house' antibody against PAT4. (B) Expression was evaluated according to the Immunoreactive score (IRS). Cytoplasmic IRS; benign SD = 1.73 and malignant SD = 3.83. Membrane IRS; benign SD = 3.13 and malignant SD = 3.66. Data provided by Dr Dan Stevens and Prof Clare Verrill (unpublished).

1.3.4 Extracellular signals in the TME: membrane-bound vesicles

Another important form of intercellular communication within the TME involves the secretion of membrane-bound vesicles known as extracellular vesicles (EVs). EV secretion is an evolutionary conserved mechanism of cell-cell communication (Deatherage and Cookson, 2012), involving the transport of ligands, receptors, cytoplasmic proteins and even nucleic acids between cells as part of normal cell homeostasis. However, more recently, EVs have been implicated in the oncogenic reprogramming of cells within the TME and the pathogenesis of numerous types of cancer (Hoshino et al., 2015; Melo et al., 2015), including PCa (Zhao et al., 2016).

1.3.4.1 EV signalling is important in PCa progression

Tumour-derived EVs play important roles in PCa progression by enabling transformed cells to thrive in the constantly evolving landscape of the TME. Specifically, EVs serve as vehicles to deliver a myriad of bioactive molecules between tumour cells and the surrounding stroma, facilitating their malignant transformation. The effects of these molecules are far-reaching and numerous; targeting cells in both the local microenvironment and in distant tissues to impact a whole host of pathophysiological processes involved in primary tumour growth and the development of secondary metastases. Consequently, due to their significant clinical potential as novel therapeutic targets and biomarkers for disease progression, there has been considerable interest in defining the mechanisms responsible for regulating the content and function of EVs in PCa.

1.3.4.2 Microenvironmental stress can regulate EV function

In order for cancer to progress, tumour cells must be able to detect and eventually overcome intracellular stress signals. Loading EVs with specific cargo capable of modifying the surrounding microenvironment is an important adaptive mechanism employed by cancer cells to combat such signals. As such, the biogenesis, composition and secretion of EVs is a tightly controlled process regulated by various stress stimuli such as hypoxia, acidosis, starvation and anti-cancer therapies (Parolini et al., 2009; Kucharzewska et al., 2013; Azmi et al., 2013). Although numerous studies have helped to highlight the potential functions of EVs secreted under numerous extracellular stress conditions, the mechanisms underpinning these changes in the secreting cells remain poorly characterised. Recently, work completed in the Goberdhan lab has identified the mTORC1-PAT4 signalling axis as an important regulator of EV signalling in colorectal cancer cells.

1.3.4.3 Exosomes: a definition

The identification of small membrane-bound vesicles within the extracellular space was first described in the 1960s, referred to then as ‘platelet-dust’ (Wolf, 1967). A couple of decades

later, the term ‘microvesicle’ started being used to describe EVs detected in biological fluids or in the conditioned medium of cultured cells (Trams et al., 1981), thought to be shed from the plasma membrane. Soon after, it was discovered that vesicles formed within intracellular compartments, known as multivesicular endosomes (MVEs), could be secreted into the extracellular environment through a process involving MVE fusion with the plasma membrane (Harding et al., 1983; Pan et al., 1985). The term ‘exosome’ was used shortly after to describe MVE-derived EVs secreted from reticulocytes (Johnstone et al., 1987), and is commonly used today to refer specifically to EVs derived from endosomal origins, although several other definitions are still used (Gould and Raposo, 2013).

1.3.4.3.1 The endocytic pathway

Exosomes are formed within the endosomal system; a discontinuous membrane transport pathway comprising several distinct membrane-bound compartments functioning together to sort internalised molecules, such as receptors, for recycling or degradation (Maxfield and McGraw, 2004). The endosomal compartments that make up this trafficking route can be distinguished based on their morphology, density, pH, and molecular signatures (e.g. Rab GTPases) into early endosomes (EEs), recycling endosomes (REs), late endosomes (LEs), and lysosomes (Dammai, 2011). The trafficking of internalised cargo through the endosomal pathway can take one of several routes depending on the cargo’s endocytic fate (Figure 1.9). For instance, all endocytosed cargo is typically delivered to the EE initially for sorting. Depending on the outcome of this sorting process, cargo is either (i) recycled back to the plasma membrane from the EE directly or indirectly via the RE; (ii) sent for degradation to the lysosome via the LE; (iii) delivered via retrograde transport to the trans-Golgi network for further sorting; or (iv) packaged into intraluminal vesicles (ILVs) in the LE and secreted as exosomes.

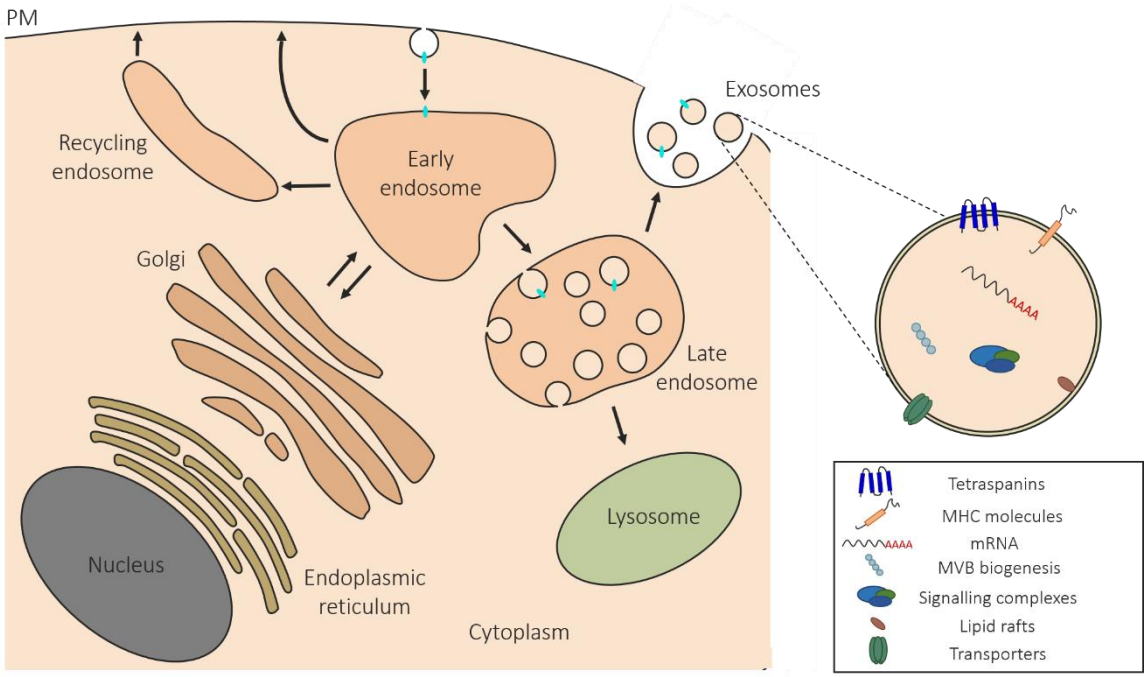


Figure 1.9. The endocytic pathway. Initially, endocytosed cargo from the cell surface is delivered to the early endosome for sorting. From here, cargo can be recycled back to the plasma membrane directly or indirectly via the recycling endosome. Conversely, cargo may be trafficked to the trans-Golgi network for sorting or targeted to the late endosome/lysosome for degradation. Alternatively, cargo packaged into ILVs in the late endosomes can be secreted as exosomes. Characteristic cargo is shown for a canonical exosome.

1.3.4.3.2 Exosome biogenesis

The description of the endosomal pathway outlined above provides an oversimplified view of what is a dynamic and complex system characterised by ongoing maturation and fusion events, both of which are essential for the proper regulation of protein traffic. For instance, EEs undergo maturation into LEs by sequestering cargo into ILVs, which then accumulate within the LE lumens through the inward invagination of the endosomal membrane. The resultant MVEs can then fuse with the acidic lysosomal compartment to enable ILV degradation, or with the plasma membrane to release the ILVs into the extracellular milieu as exosomes (Jaiswal et al., 2002, Raposo et al., 1996). Although the mechanisms that regulate MVE fate remain unknown, different populations of MVEs have been identified (Mobius et al., 2002), and are believed to be formed via distinct biogenesis pathways

(Buschow et al., 2009; Babst et al., 2011; Van Niel et al., 2011). Whilst the best characterised machinery known to drive ILV formation is the endosomal sorting complexes required for transport (ESCRT), recent studies have revealed that ESCRT-independent pathways also exist (Trajkovic et al., 2008; Stuffers et al., 2009).

ESCRT-dependent exosome biogenesis involves the sequential recruitment of the ESCRT machinery to the surface of the endosomal membrane to orchestrate different steps in the formation of ILVs (Figure 1.10). Considering exosomes correspond to ILVs, the same mechanisms are thought to underpin exosome biogenesis. The ESCRT machinery comprises a group of evolutionary conserved proteins that assemble into four multi-protein complexes termed ESCRT-0, -I, -II and -III, along with several accessory proteins (Hanson and Cashikar., 2012). The first step in ILV formation begins with the recruitment of ESCRT-0 to the endosomal membrane, through interactions with lipids and ubiquitinated cargoes (Ren and Hurley, 2010). From here, ESCRT-0 recruits TSG101, a subunit of the ESCRT-I complex, which, in turn, recruits ESCRT-III via ESCRT-II or the accessory protein Alix (Babst et al., 2002). Together, ESCRT-I and II initiate the inward budding of the limiting endosomal membrane, and ESCRT-III subsequently acts to constrict, and ultimately sever, the neck of newly-developed membrane bud (Wollert et al., 2009). Finally, ESCRT-III recruits auxiliary factors like the AAA-ATPase VPS4 to disassemble the ESCRT complex (Hurley, 2010), completing the process. Whilst the ESCRT machinery plays an essential role in ILV formation in yeast (Odorizzi et al., 1998; Nickerson et al., 2010), the organism in which this mechanism was first elucidated, the role of this complex in mammalian cells remains contentious.

ESCRT-independent exosome biogenesis is the process of ILV formation via mechanisms which do not involve the ESCRT machinery. Evidence for this pathway includes the finding that mammalian cells maintain the ability to form ILVs, and thereby exosomes, even in the absence of key ESCRT components (Stuffers et al., 2009). Alternatively, distinct lipid

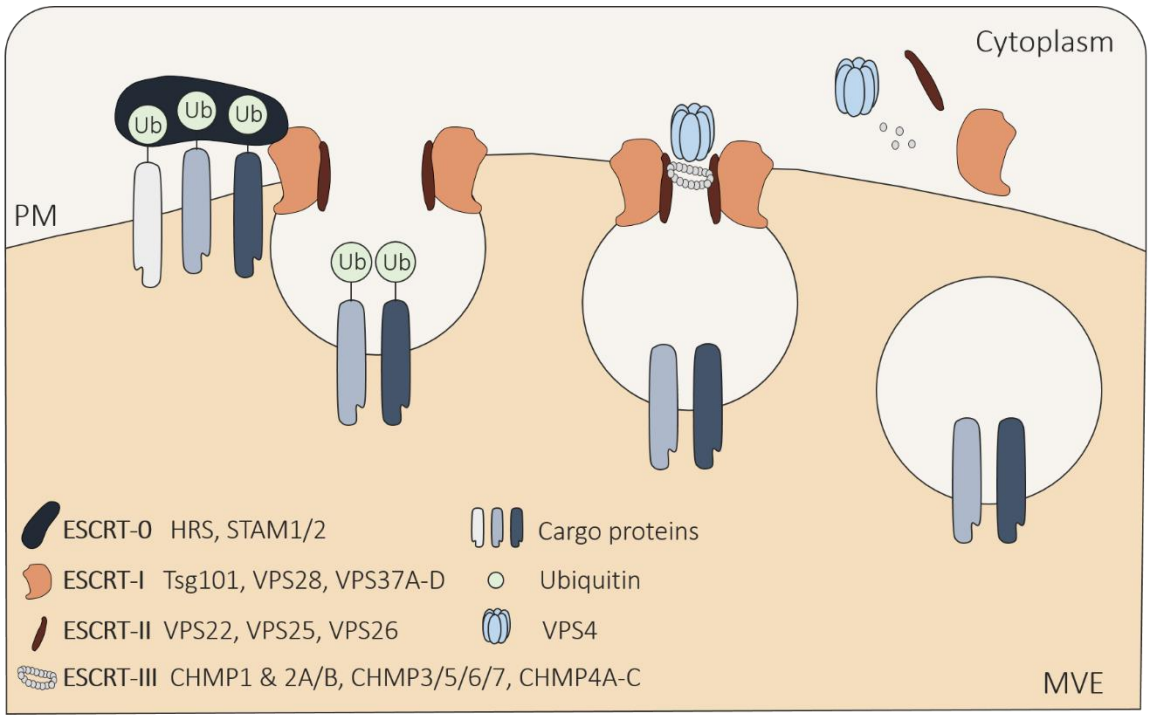


Figure 1.10. ESCRT-dependent ILV formation. The ESCRT complex is recruited to the cytosolic side of the MVE membrane. Endocytosed cargo to be sorted into ILVs, such as cell-surface receptors, are ubiquitinated in their cytosolic tail and then recognised and sequestered by the ESCRT-0 complex. ESCRT-I then binds to ESCRT-0 and recruits the ESCRT-II complex. Together, the ESCRT-I and -II complexes promote the inward bending of the endosomal membrane and the shuttling of sorted cargo into the newly-formed bud. ESCRT-II then recruits and initiates the oligomerisation of the ESCRT-III components around the neck of the ILV, and is subsequently disassembled by the v-AAATPase VPS4 to drive vesicle scission.

microdomains may function in such settings to induce membrane bending and ILV formation instead (Kooijman et al., 2003; Staneva et al., 2009). Indeed, using an *in vitro* membrane model, it was shown that liposomes which contain lysobisphosphatidic acid (LBPA) require only a pH gradient to form ILVs (Matsuo et al., 2004), supporting such a theory. Also supporting a role for lipids in ILV formation is the observation that exosomes typically contain high concentrations of ceramide, and that inhibiting ceramide production reduces exosome formation and release (Trajkovic et al., 2008). However, similar findings have not been reported in other cell lines, such as melanoma cells (van Niel et al., 2011), where exosome biogenesis may involve the tetraspanin protein CD63 instead. Indeed, in

addition to lipids, domains enriched for tetraspanins have been also been proposed to function in ESCRT-independent exosome biogenesis (Perez-Hernandez et al., 2013).

Nonetheless, despite the many studies demonstrating exosome formation in the absence of ESCRT machinery, several others support a role for ESCRTs in this process. For instance, many ESCRT components and ubiquitylated proteins have been identified in exosomes isolated from various cell types (Théry et al., 2001; Buschow et al., 2005). Furthermore, a number of gene silencing studies have implicated several ESCRT and accessory proteins in the formation and sorting of cargo into ILVs that are released as exosomes (Tamai et al., 2010; Baietti et al., 2012; Colombo et al., 2013). Hence, unlike the system established in yeast, in higher eukaryotes, it is likely that ESCRT-dependent and -independent mechanisms of ILV formation exist. Moreover, it is thought that these pathways may not function entirely separately but, in some cases, work synergistically to regulate exosome biogenesis (Maas et al., 2017).

1.3.4.3.3 Exosome heterogeneity

The diverse mechanisms involved in ILV formation in mammalian cells indicates that the regulatory mechanisms underpinning exosome biogenesis may be more extensive and, therefore, more complex than once believed. These observations further highlight that cells contain subpopulations of exosomes that are controlled by different machineries, and that these machineries may vary depending on the cell type or physiological conditions. Hence, the heterogeneity that can exist within this EV population may be enormous. Indeed, exosomes distinct in size, cargo, and lipid composition have been isolated from the same cell lines. However, such heterogeneity exists not only in the mechanisms controlling the formation of exosomes, but in the identity of the MVEs also. Similar to exosomes, subpopulations of MVEs coexist simultaneously within cells (Möbius et al., 2003; Colombo et al., 2014), with some destined for degradation and others for exocytosis. Typically, MVEs fated for either pathway carry proteins representative of their endocytic parentage, which is

classically believed to be the LE (Jaiswal et al., 2002, Raposo et al., 1996), and therefore includes proteins such as the small GTPase Rab7 (Russell et al., 2006). However, secreted MVEs bearing markers of the EE have also been reported (Vidal and Stahl, 1993). Furthermore, work recently completed by members of the Goberdhan lab and collaborators revealed that exosomes can form within, and be secreted from, endocytic compartments carrying Rab11, a marker of the RE, in a mechanism conserved between *Drosophila* and human cancer cells (Fan et al, *under revision*). Together, these findings suggest that compartment identity may represent an additional level of MVE heterogeneity and challenge the classical paradigm of exosome biogenesis in the endocytic pathway that is: EE>LE>MVE>exosome.

1.3.4.3.4 The exosome switch model

Although a number of studies published in recent years have highlighted that different MVE subpopulations exist and contribute to exosome heterogeneity, the role distinct MVEs play in cell biology remains unknown. In general, it is likely that multicellular organisms utilise this strategy - to compartmentalise ILVs into different MVE subpopulations - in order to rapidly coordinate changes in cellular responses. Indeed, distinct subpopulations undoubtedly offer cells greater control over their behaviour, enabling them to fine tune which MVE populations to degrade or secrete depending on the desired response. Interestingly, changes in extracellular conditions have recently been shown to affect the fate of different MVE subpopulations. For instance, in immature dendritic cells (DCs), ubiquitinated MHC class II proteins are typically sorted into MVEs for degradation. However, in the presence of antigen-carrying T cells, DCs generate a population of smaller MVEs enriched for MHC class II molecules that are exocytosed, releasing exosomes that collect at the surface of T cells and may function in the immune response (Buschow et al., 2009). Likewise, epidermal growth factor (EGF) stimulation has been shown to generate at least two distinct MVE subpopulations in HeLa cells; one positive for the EGF-receptor and another devoid of the receptor and likely fated for degradation (White et al., 2006). Moreover, in human epithelial

type 2 (HEp-2) cells, EGF has been shown to induce ESCRT-dependent ILV formation (White et al., 2006), whereas others have reported that ILVs are usually formed via ESCRT-independent mechanisms in this cell line (Stuffers et al., 2009). Hence, EGF signalling may be able to regulate the production of distinct MVE subpopulations and the type of ILVs formed within them, presumably to produce a more specific cellular response.

Interestingly, signalling via the growth factor-regulated mTORC1 pathway may control the fate of Rab11-marked MVEs in HCT116 colorectal cancer cells. Work completed in the Goberdhan lab has shown that suppression of mTORC1 activity via glutamine depletion or PAT4/raptor knockdown increases the secretion of exosomes derived from this compartment (Shih Jung Fan, Sumeth Perera; unpublished data). Moreover, this effect was shown to be coupled with increased protein trafficking via Rab11-marked endosomes, and decreased flux through - and exosome secretion from - the classical Rab7-CD63-positive LE pathway (Fan et al, *under revision*). Findings further revealed that exosomes secreted under glutamine deprivation could promote tumorigenic effects in recipient cells, and that this response was blocked by silencing of Rab11 in the secreting cells (Shih Jung Fan, Cleidson Alves; unpublished data). Hence, by altering the endocytic route of exosome biogenesis, so-called ‘exosome switching’, colorectal cancer cells may be able to alter the behaviour of cells within the TME and overcome metabolic stress.

Nonetheless, it remains unknown whether this switching mechanism is a specific adaptation of HCT116 cells or is employed by other cancer cell types, and furthermore, whether this response is regulated by microenvironmental stressors besides nutrient deprivation. Hence, the scope of the exosome switching event is yet to be realised.

1.3.4.3.5 Challenges in exosome research

Normal cells and cancer cells secrete a whole host of EVs. In addition to exosomes, the two other most prominent EV types include microvesicles and apoptotic bodies (Figure

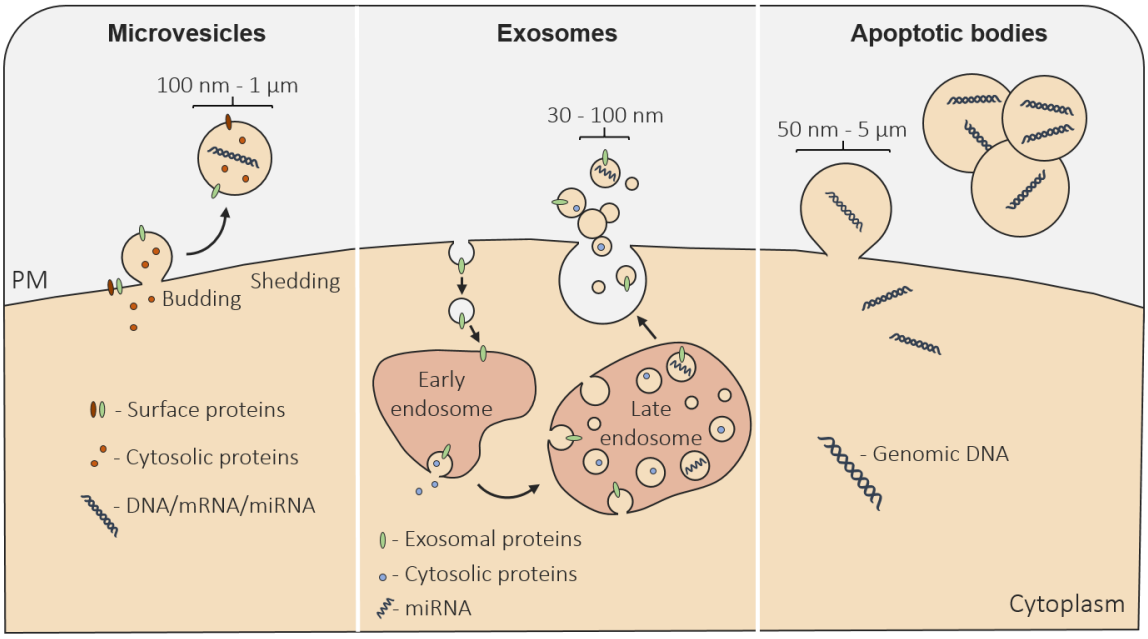


Figure 1.11. EV heterogeneity. EVs can be divided into three main populations according to their route of biogenesis: exosomes, microvesicles and apoptotic bodies. Exosomes are produced via the fusion of MVEs with the plasma membrane, whereas microvesicles and apoptotic bodies are formed via the budding or blebbing of the cell membrane. EVs can also be characterised according to their molecular composition and biophysical features: exosomes measure between 30 – 100 nm, microvesicles between 100 nm –1 µm, and apoptotic bodies up to 5 µm. EVs contain numerous proteins, lipid, and nucleic acids which can differ between the three populations; however, as yet, no markers have been identified allowing for clear discrimination between the vesicle subtypes following isolation. Diagram adapted from Journal of Endocrinology 228 R57-R71.

1.11). Vesicles are classified into one of these three groups based upon their mode of biogenesis. For instance, exosomes are produced by the fusion of MVEs with the cell surface; microvesicles are generated by the direct outward budding and fission of the plasma membrane; and apoptotic bodies are released following fragmentation of the plasma membrane during the apoptotic process. Hence, cells secrete a heterogeneous population of EVs, within which additional EV subpopulations exist (e.g. exosomes derived from different endocytic compartments). However, the field currently lacks tools to isolate vesicles based upon their routes of biogenesis; therefore, differences in the biophysical properties (e.g. size, density) of individual EV populations are exploited instead.

Consequently, vesicles that share similar characteristics typically co-isolate, resulting in the purification of a heterogeneous population of EVs instead.

1.3.4.3.6 Exosome isolation techniques

A number of different EV isolation methods have been developed, the most common of which include ultracentrifugation (UC), ultrafiltration (UF), and size-exclusion chromatography (SEC). However, the basis upon which vesicles are separated and the ability of each technique to isolate pure EV populations can vary considerably. In differential UC (dUC), the most commonly used method of exosome isolation, vesicles are separated depending on the speed at which they pellet under different centrifugal forces (Koh et al., 2018). In theory, large EVs (e.g. apoptotic bodies) are pelleted by centrifugation at 2,000 × *g*; microvesicles at 10,000 × *g*; and exosomes at forces of 100,000 × *g* and above. Using dUC, characterisation studies have revealed that ESCRT proteins and tetraspanins are typically enriched for in the exosomal pellet and not in the microvesicle pellet (Menck et al., 2013), supporting the efficacy of this technique. However, distinct EV types of similar size, as well as protein aggregates, can also co-sediment at 100,000 × *g* indicating that dUC enables the enrichment, but not purification, of different EV populations (Théry et al., 2006). Indeed, this was highlighted in a recent study that showed certain exosomal markers were enriched for at speeds higher than 100,000 *x g,* indicating that dUC does not allow for separation of distinct exosome subpopulations (Jeppesen et al., 2014).

Density gradient UC is a technique commonly used to combat the issues frequently encountered using dUC. In this method, particles are separated based upon their rate of migration through a density gradient of viscous solution (e.g., sucrose or iodixanol). Factors that affect this rate include particle size, shape, and density; hence, protein aggregates typically sediment when placed in such a gradient, whereas lipid-containing vesicles float to a position of equilibrium buoyant density. Therefore, using this method, EVs can be

separated from protein contaminants as well as other EV populations, even subpopulations. Indeed, studies that have applied this technique have hinted at the existence of multiple, distinct exosome subtypes in a number of cell types based upon differences in their biophysical features and molecular compositions. For instance, distinct exosome subpopulations have been identified in exosomes isolated from seminal plasma that share similar equilibrium buoyant densities but differ in their overall size and protein composition (Aalberts et al., 2012). Conversely, in mouse breast cancer cells, exosome subpopulations that vary in their biophysical properties but are enriched for the same protein markers have been discovered (Bobrie et al., 2012). As have subpopulations that exhibit similar biophysical properties yet are secreted via different regulatory pathways (Bobrie et al., 2012). Hence, density gradient UC represents a powerful technique for studying exosome heterogeneity.

Although dUC and density gradient UC remain the most commonly used methods for EV isolation, with the latter proving useful in the isolation of distinct EV subpopulations, both present considerable disadvantages. Experimentally, both techniques are time-consuming, lack automatization, and frequently generate low or operator-dependent yields. Moreover, EVs isolated via UC reportedly exhibit lower functionality, likely due to vesicle sheering or aggregation resulting from high speed centrifugation (Mol et al., 2017). Consequently, a number of easy-to-use and, in some cases, more effective, alternatives have been developed, two of which include UF and SEC.

In UF, EVs are separated based upon their ability to pass through a semi-permeable membrane with a defined pore size. Particles larger than the membrane filter are retained, whereas smaller particles elute and are discarded. Hence, by varying the pore size, in theory, one can enrich for different EV populations. For instance, one study demonstrated that EVs obtained after conditioned supernatant from LIM1863 colon cancer cells was filtered through a membrane with a very small pore size (0.1 µm), and then centrifuged at

100,000 x g, were strongly enriched for exosomal markers (e.g. Tsg101, Alix, syntenin). Conversely, EVs derived from passing the supernatant through a larger filter (0.65 µm) and pelleting at a slower speed (10,000 x g) were enriched for proteins indicative of microvesicles instead (Xu et al, 2015). Hence, UF may allow for a more straightforward isolation of different EV populations compared to UC. Also, if used as a standalone technique, UF may minimise the damage to EVs encountered during dUC and better preserve vesicle function (Willms et al., 2018). Nonetheless, UF remains a very crude method of EV isolation. Like with dUC, particles of similar sizes co-purify, including different EV populations as well as protein aggregates.

The use of SEC to isolate EVs has become increasingly popular in recent years and is even preferred over UC by some groups. In SEC, EVs are separated based upon their rate of migration through a column filled with porous resin with defined pore sizes. As the EV-containing solution is passed through the resin, small particles (e.g. protein) enter the pores and large particles (e.g.EVs) are excluded. Consequently, larger particles travel through the column faster and elute in earlier fractions. Studies using SEC have reported that EVs isolated using this method share similar morphological features to those purified via UC and are enriched for a similar set of exosomal proteins, indicating that both methods may isolate the same population of vesicles (Mol et al., 2017). However, more recent studies have shown that SEC can also be used to separate different EV subpopulations. For instance, using specialised column resin with larger pore sizes, coupled with density gradient UC, two distinct exosome subpopulations displaying different enrichment profiles were identified in exosomes derived from B16F10 melanoma cells (Willms et al., 2016). A similar methodology was used to reveal two exosome subpopulations of distinct size and protein composition in EV preparations isolated from human mesenchymal stem cells (Willis et al., 2017). Hence, isolation of distinct exosomes subpopulations appears to be achievable with SEC, and may offer a user-friendly alternative to density gradient UC for the study of EV heterogeneity.

As discussed, increased secretion of an alternative population of exosomes derived from Rab11-marked endocytic compartments is a feature of the exosome switch as characterised in HCT116 cells. However, it remains unknown whether subpopulations of these 'switched' exosomes exist, as most work completed in the host lab so far has utilised dUC for the purposes of vesicle isolation. Furthermore, if such subpopulations do exist, an important aim will be to determine whether mTORC1 signalling differentially regulates distinct exosome subtypes. Indeed, we must first identify both the (i) EV subtypes present within Rab11-marked compartments and (ii) the signalling mechanisms by which they are regulated, before we can fully appreciate the role of exosome switching in disease.

1.4 Aims and objectives

Despite the increasing interest in targeting the TME in advanced PCa, there are many key outstanding questions that have yet to be answered. In particular; what intercellular signals are important in regulating PCa cell growth within this environment? Indeed, only once such signals have been identified, and their roles in PCa progression characterised, can effective therapeutic strategies be developed. My work presented in this thesis addresses this question by investigating the effects of BMPs (Chapter 3), nutrient starvation (Chapter 4), and hormone-deprivation (Chapter 5) on PCa cell growth directly, and indirectly via EVs. I focus primarily on the regulatory pathways responsible for mediating the downstream effects of these signals in a number of different PCa cell lines *in vitro*. Overall, the aims of this thesis include:

Aims:

i. Characterise the effects of BMP signalling on PCa cell behaviour
ii. Determine the role of the PAT4-mTORC1 signalling axis in PCa cells
iii. Establish whether the mTORC1 pathway regulates distinct EV subtypes

The resulting work has provided novel insight not only into our fundamental understanding of PCa cell biology, but also the ways in which these behaviours may contribute to PCa pathogenesis. In discussing my data, I will highlight the clinical implications of my findings, along with the potential avenues for therapeutic intervention in light of these new discoveries.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Methods

2.1.1 Prostate cancer cell characterisation

2.1.1.1 Cell culture

All PCa cell lines used in this project (Table 2.1) were purchased from ATCC, except for the C4-2B cells, which were a generous gift from Dr Richard Bryant (John Radcliffe Hospital, University of Oxford). HEK293T cells were also purchased from ATCC and the bone stromal cell lines HS-5 and HS-27a were a kind gift from Dr Claire Edwards (Botnar Research Centre, University of Oxford). LNCaP, C4-2B, HS-5 and HS-27a cells were maintained in RPMI-1640 medium (Gibco®, Invitrogen); VCaP cells in high glucose DMEM (Gibco®, Invitrogen); DU145 cells in Eagle's Minimum Essential Medium (Gibco®, Invitrogen); PC-3 cells in DMEM/F12 (Gibco®, Invitrogen), and HEK293T cells in DMEM with GlutaMAX™ (Gibco®, Invitrogen). All media was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies) and 100 U/ml Penicillin-Streptomycin (Life Technologies). All cells were incubated at 37°C with 5% CO_2 and were not kept past passage 25.

	LNCaP	C4-2B	PC-3	DU 145	VCaP
Site of origin	Lymph node	Bone	Bone	Brain	Vertebrae
AR expression	Mutant	Mutant	-	-	WT
Androgen stimulation	Increases growth	No change in growth	No change in growth	No change in growth	Increases growth
Doubling time	~60 hrs	~48 hrs	~33 hrs	~34 hrs	~120 hrs
PTEN status	-/-	-/-	-/-	+/-	+/+
Rb expression	WT	WT	WT	-	WT

Table 2.1. Overview of the PCa cell lines used in this study (Cunningham and You, 2015)

2.1.1.1.1 Comparison of culture media on PCa cell growth and mTORC1 activity

PCa cells were plated in complete growth medium (i.e. plus 10% FBS) and incubated overnight. The next day, culture media was replaced with fresh complete growth medium (standard conditions) or with a basal formula (i.e. without 10% FBS) supplemented with 1% Insulin-Transferrin-Selenium (ITS) to emulate EV isolation conditions. To analyse differences in cell growth, phase contrast images were collected every four hours for a total of 48 hours using the IncuCyte® ZOOM live-cell imager, and cell confluency was calculated using the IncuCyte™ image analysis software. Protein lysates were collected after 24 and 48 hours to assess alterations in mTORC1 activity via Western blotting.

2.1.1.1.2 Androgen and anti-androgen treatment

Cells were plated in their basal culture medium supplemented with 10% charcoal-stripped serum (CSS; Gibco®, Invitrogen). The following day, cells were treated with indicated concentrations of DHT, alone, or in combination with bicalutamide (concentrations indicated throughout text) and control cells were treated with equal volumes of vehicle (MeOH + DMSO) for 24-96 hours.

2.1.1.1.3 mTORC1 inhibition

Cells were plated in standard culture medium supplemented with 10% FBS. Media was changed the next day to a serum-free formula plus 1% ITS (Gibco®). At this point, cells were treated with indicated concentrations of the small molecule inhibitors Torin1 (SelleckChem) or rapamycin (Sigma), and control cells with equal volumes of vehicle (DMSO) for 24 hours.

2.1.1.1.4 Glutamine depletion

Cells were plated in complete growth medium and left to attach overnight. The following day, media was changed to DMEM/F12 without L-glutamine (Gibco®, Invitrogen),

supplemented with indicated concentrations of glutamine (Gibco®, Invitrogen) and 1% ITS. Control cells were treated with an equal concentration of glutamine to that found in standard DMEM/F12 media (15 mM), and all cells were incubated for 24 hours.

2.1.1.1.5 BMP-6 stimulation

PCa cells were plated in basal culture media supplemented with 10% CSS. Following overnight incubation, cells were stimulated with indicated concentrations of BMP-6 and control cells were treated with equal volumes of vehicle (0.1% BSA) for 3-96 hours.

2.1.1.2 Genetic manipulation of PCa cell lines

2.1.1.2.1 DNA purification

Transformed bacteria, from glycerol stocks or stab culture, were streaked onto LB Agar plates (tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, pH adjusted to 7.2; autoclaved) containing selective antibiotic and left to incubate overnight (12-18 hours) at 37°C, whilst shaking. The following day, a single colony was picked and transferred into 3 mL LB medium (tryptone 10 g, yeast extract 5 g, NaCl 10 g, pH adjusted to 7.2; autoclaved) containing the appropriate selective antibiotic for 8 hours at 37°C on a shaker. The starter culture was then diluted into 250 mL selective LB medium and grown for 12–16 hours at 37°C, whilst shaking. The next day, bacterial cells were harvested by centrifugation (6000 *x g*; 15 mins; 4°C) and DNA purification was carried out using the QIAGEN® Plasmid Maxi Kit, according to the manufacturer's instructions. Purified DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (New Brunswick) and stored at -20°C. All plasmids used are listed in Table 2.2.

2.1.1.2.2 Transient transfections

LNCaP (2 x 10^5 cells/well) and C4-2B (1.8 x 10^5 cells/well) cells were seeded into 12 well plates and left to attach overnight. Per well, the following reactions were setup: 1 µg of DNA was diluted in 200 µl Opti-MEM® I reduced serum medium (Gibco®, Life Technologies) and to this, 2 µl of Lipofectamine® 2000 reagent (Life Technologies) was added and left to incubate for 25 minutes at room temperature. Prior to transfection, each well was replaced with fresh, complete growth medium. The DNA-Lipofectamine® solution was then added and allowed to incubate with the cells overnight at 37°C. The media was changed again to complete growth formula the following morning.

2.1.1.2.3 NucLight Red stable cell lines

LNCaP (5 x 10^5 cells/well) and PC-3 (2.5 x 10^5 cells/well) cells were seeded into 6 well plates and allowed to attach overnight. The following day, cells were transduced with NucLight Red lentivirus reagent (IncuCyte®, EssenBio) at a multiplicity of infection (MOI = TU/cell) of 3 in the presence of 8 µg/ml polybrene (Sigma), then incubated for 24 hours at 37°C. Culture media was replaced the next day with fresh complete growth medium, and replaced again 48 hours post-transduction, to include Zeocin (Invitrogen) at a concentration of 100 µg/ml for LNCaP cells and 200 µg/ml for PC-3 cells. NucLight red-expressing LNCaP and PC-3 cells were maintained in complete growth medium supplemented with 50 µg/ml and 100 µg/ml Zeocin, respectively.

2.1.1.2.4 PAT4 knockdown

A total of 5 x 10^5 HEK293T cells per well were seeded into 6 well plates and allowed to settle overnight. The next day, lentiviral vectors expressing the shRNA targeting PAT4 were produced as follows: HEK293T cells were co-transfected with shRNA-PAT4 plasmid DNA, along with psPAX2 packaging and pMD2.G envelope plasmid DNA at a ratio of 4:3:1, respectively. Specifically, 0.5 µg shPAT4, 0.375 µg psPAX2 and 0.125 µg pMD2.G plasmid DNA was added to 150 µL Opti-MEM® I reduced serum medium and combined with 4 µL GeneJuice® transfection reagent (Merck Millipore). The mixture was incubated at room temperature for 30 minutes and then added to each well in a dropwise manner. Approximately 24 hours post-transfection, the medium was replaced with complete growth media and left to incubate for a further 24 hours prior to the initial collection of the conditioned medium. An additional collection was made after a further 24 hours. The conditioned media from the two collections were then combined and cleared by centrifugation (500 *x g*, 4°C, 5 minutes) and filtration through a 0.45 µm pore PVDF Millex-HV filter (Merck Millipore). Finally, the lentiviral media was added to VCaP cell cultures (seeded at a density 7.5 x 10^5 cells per well into 6 well plates, three days prior) at indicated

volumes, along with 8 µg/ml polybrene. Following overnight incubation, lentiviral media was removed, and fresh complete growth medium was added.

2.1.1.2.5 Rb knockdown

Two pLKO-based shRNA constructs targeting different sequences in human RB1 mRNA were used to knockdown Rb (Table 2.2). NucLight Red-expressing LNCaP cells were transfected with either a non-targeting (shNT) control plasmid or one of the plasmids targeting Rb (shRB) using Lipofectamine®. Forty-eight hours post-transfection, cells were selected with 2 µg/mL puromycin until all non-transfected cells were dead (~6 to 7 days). A mixed population of stable NucLight red-expressing, Rb knockdown LNCaP cells were maintained in complete growth medium containing 0.5 µg/mL puromycin. Western blotting and RT-PCR assays were used to confirm loss of Rb mRNA and protein.

Insert	Vector backbone	Vector type	Selection	Source
RB1-shRNA 63	pLKO.1 puro	Lentiviral	Puromycin	Addgene (#25641)
RB1-shRNA 19	pLKO.1 puro	Lentiviral	Puromycin	Addgene (#25640)
ShNT	pLKO.1 puro	Lentiviral	Puromycin	Sigma
PAT4-shRNA	pLKO.1 puro	Lentiviral	Puromycin	Sigma
VSV G	pMD2.G	Lentiviral	N/A	Addgene (#12259)
Gag, Pol, Rev, Tat	psPAX2	Lentiviral	N/A	Addgene (#12260)
CD63-GFP	pCT	Lentiviral	N/A	Kind gift of the Wood lab
ARE-Luc	pGL3	Mammalian Expression	Ampicillin	Kind gift of the Rinaldi lab
Ren-Luc	pRL-TK	Mammalian Expression	Ampicillin	Kind gift of the Rinaldi lab

Table 2.2. List of plasmids used in this study

2.1.2 Isolation and characterisation of EVs

2.1.2.1 Isolation conditions

For EV isolation, cells were plated in complete growth media at the following densities; 2.5 x 10^7 VCaP cells per dish, 1.2 x 10^7 LNCaP cells per dish and 1 x 10^7 C4-2B cells per dish in 150 mm dishes; (12 dishes/condition). Cells were left to attach overnight and had reached approximately 80% confluency before beginning EV collection. Generally, serum-free media conditions were used when isolating EVs to eliminate contaminating EVs present in FBS. Instead, standard growth medium was supplemented with 1% ITS, unless otherwise indicated. However, for EV isolation from VCaP cells following anti-androgen treatment, medium was supplemented with 1% pre-spun CSS instead of ITS. Briefly, CSS was centrifuged for ~18 hours (100,000 *x g*; 4°C) and the EV-depleted supernatant was filtered through a 0.22 µm pore PVDF Millex-HV filter (Merck Millipore) and analysed by nanosight tracking analysis, transmission electron microscopy and western blotting before being added to media. Cells were incubated in the isolation media for a period of 24 hours and EVs were purified from the conditioned media using one of three methods (Figure 2.1).

2.1.2.2 EV isolation

2.1.2.2.1 Size-exclusion chromatography (SEC)

After 24 hours, conditioned medium was collected and pre-cleared by centrifugation (500 *x g*, then 2,000 *x g*; 10 minutes each, 4°C) to remove whole cells and cell debris. The supernatant was subsequently vacuum filtered through a 0.22 µm pore membrane to eliminate any larger vesicles of non-endosomal origin. The resultant filtrate was concentrated approximately 25-fold using the Vivaflow 50 R tangential flow filtration (TFF) system (Sartorius, MWCO = 100 kDa) and further concentrated to a final volume of 1 ml by repeated centrifugation (4000 × *g*, 4°C) using an Amicon Ultra-15 spin-filter unit (Merck Millipore, MWCO = 100 kDa). The sample was then loaded onto a 24 ml SEC column

packed with sepharose CL-4B (GE Healthcare Life Sciences) and eluted as 1 ml fractions in PBS (Gibco®, Invitrogen). For EV analysis, select fractions (indicated in text) were pooled together and concentrated to approximately 100 µl via ultrafiltration, then stored at -80°C.

2.1.2.2.2 Ultrafiltration

Cell culture supernatant was harvested and then pre-cleared by sequential centrifugation and filtration (details above). The filtrate was subsequently loaded onto Amicon Ultra-15 centrifugal filter units (MWCO = 100 kDa) and centrifuged for 15 minutes (4000 × *g*, 4°C). Afterwards, the sample was washed twice with PBS to minimise soluble protein contaminants and concentrated to approximately 100 µl by repeated centrifugation (4000 × *g*, 4°C). Samples were stored at 4°C prior to analysis.

2.1.2.2.3 Differential ultracentrifugation

Conditioned media was harvested subjected to the same initial pre-clearing centrifugation and filtration steps as detailed above. Subsequently, the filtrate was concentrated using the Vivaflow 50 R TFF system (MWCO = 100 kDa) and then centrifuged for 2 hours (120,000 *x g*; 4°C) to pellet EVs using a fixed-angle Type 55.2Ti rotor (Beckman Coulter). Afterwards, the supernatant was poured off and EVs were re-suspended in PBS. Ultracentrifugation was then repeated (120,000 *x g*; 2 hours; 4°C) in an attempt to reduce the amount of contaminating proteins present in the sample. Finally, the EV pellet was re-suspended in approximately 100 µl PBS and stored at -80°C.

2.1.2.2.4 Sucrose density UC

Exosomes isolated via DUC were loaded on top of a discontinuous sucrose gradient of 10, 1 ml fractions ranging from 2.5 M to 0.4 M sucrose (Sigma) in HEPES buffer (pH 7.4,

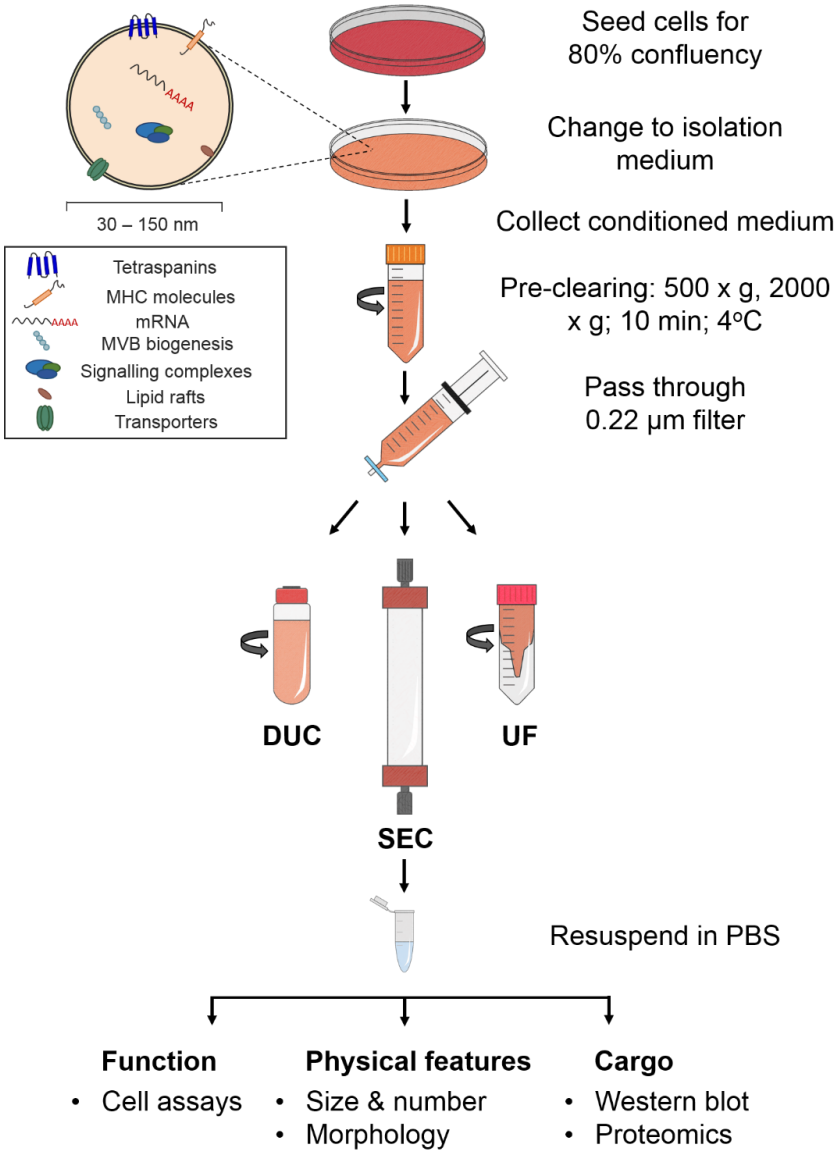


Figure 2.1. The main strategies used for EV isolation and characterisation in this study

Sigma). Samples were centrifuged for 16 hours (200,000 × *g*, 4°C) in a SW41 swinging bucket (Beckman Coulter), and afterwards each 1 mL fraction was collected and its average density (g ml^{-1}) calculated. EVs were purified from each fraction by ultracentrifugation (110,000 *x g,* 70 min, 4°C) and then washed in PBS (110,000 *x g,* 70 min, 4°C).

2.1.2.3 EV characterisation

2.1.2.3.1 Nanosight Tracking Analysis (NTA)

EV particle size and concentration data were obtained using the NanoSight NS500 instrument (Malvern, UK) equipped with a 405 nm laser, CCD camera and NTA software version 2.3 (NanoSight Ltd.). Samples were diluted in PBS to a concentration between 2 × 10^8 and 10 × 10^8 particles/ml. All measurements were performed using identical camera settings (camera level = 13). Camera focus was adjusted for each sample to ensure that most of the particles appeared as single, defined dots. Once EV samples were loaded into the sample chamber, three videos of 30 seconds each were analysed using the following script: PRIME, DELAY 7, CAPTURE 30, REPEAT 3. Recordings were used to generate graphs plotting EV concentration (particles x 10^8/ml) vs. size distribution (nm). All post-acquisition settings were set to automatic (except for the detection threshold which was set to 5) and kept constant between samples.

2.1.2.3.2 Transmission electron microscopy (TEM)

All TEM work was completed in collaboration with Dr Errin Johnson at the Dunn School Bioimaging Facility (Sir William Dunn School of Pathology, University of Oxford). A 5–10 µl drop of EV suspension (~0.2 mg/ml) was adsorbed onto a glow discharged carbon-coated copper grid (300 mesh, 3 mm grid, TAAB Laboratories) for approximately 2 minutes. After adsorption, grids were blotted dry with filter paper and washed with Milli–Q water. To negatively stain the EVs, uranyl acetate (2% w/v) was applied to the grids for 10 seconds, before the excess solution was removed with clean filter paper. The grids were then air dried and imaged on a FEI Tecnai12 TEM (FEI UK Ltd) operating at an accelerating voltage of 120 kV. Images were captured using a bottom-mounted Gatan OneView camera at 120,000x magnification.

2.1.2.3.3 Immunodot blot analysis

A total 0.5 ml of the 1 ml fractions separated via SEC were spotted onto nitrocellulose membranes (Merck Millipore) using a dot blot apparatus (Bio-Rad). Samples were allowed to dry before the membrane was blocked in 5% skimmed milk in TBST, and incubated overnight at 4°C. Afterwards, the membranes were processed and imaged the same as for Western blotting (see above).

2.1.2.3.4 Trypsin digestion assay

EVs isolated via ultrafiltration were quantified using the BCA Protein Assay Kit (Thermo Scientific Pierce). A total of 5 µg of sample was used in each of the following reactions: untreated EVs, EVs incubated with 50 µg/ml trypsin (Gibco®, Invitrogen) for 30 minutes or one hour at 37 °C, or EVs treated with 0.1% Triton X-100 (Sigma) for 5 minutes at 4°C and then trypsin for 30 minutes or one hour at 37 °C. Subsequently, the samples were processed for Western blotting (details above) and analysed for Syntenin-1 and AR protein expression to determine their resistance to trypsin digestion.

2.1.2.3.5 EV normalisation

To compare changes in the protein profile of EVs secreted from control vs. treated cells via western blotting, a method of EV normalisation developed by Dr Shih Jung Fan (Goberdhan lab, DPAG, University of Oxford) was employed. As the treatments being used here to study the regulation of EVs are known to also regulate cell growth, the following calculation is an attempt to account for any discrepancy in growth by normalising the volume of the EV sample loaded to the total protein content of the cells, relative to control. Hence, the differences observed should reflect alterations in EV regulation, rather than the number of secreting cells.

Essentially, the basis for this normalisation method is given by the following equation

describing the exponential growth of cells:

$$\mathrm{Log}(N) = \mathrm{Log}(N0) + \mathrm{K\,t}$$

Where the increase in the growth of the cells *(N)* is proportional to the number of cells present in the culture at the beginning of the time interval *(N0)* plus the growth constant (K) multiplied by the unit time (t).

For this work, total protein content was used as a proxy for cell number. Hence, the rate of cell growth was indirectly calculated by quantifying the total protein content of cells at 0 hours and then at 24 hours. Briefly, when plating cells for EV isolation, two additional dishes were plated in parallel, and served as the 0-hour controls *(N0)*. Before the cell culture medium was changed to begin EV collection, these plates were lysed, and their average protein content determined using the BCA Protein Assay Kit. Following a 24-hour period of EV collection, an additional three plates per condition were lysed and the average protein content was calculated for the 24-hour control and treated samples *(N)*.

Since t is a constant across all experiments (i.e. 24 hours), the EV normalisation factor could then be determined by calculating the ratio between two growth constants:

$$\frac{Ka = Log(Na) - Log(N0a)}{Kb = Log(Nb) - Log(N0b)}$$

As isolated EVs were resuspended in equal volumes of PBS, the normalisation factor could be applied to directly adjust the amount of treated sample used for Western blotting, relative to control.

2.1.2.3.6 EV functional analysis: prostate cancer cell growth

LNCaP cells (6000 cells/well) were seeded into 96 well plates in 100 µl of their standard culture medium supplemented with 1% or 10% CSS. Cultures were incubated for 24 hours

to allow cells to successfully attach to the bottom of each well. At this point, EVs freshly isolated via UF were quantified using the NTA. A total of 1 x 10^3 or 10 x 10^3 particles/cell were then mixed with 100 µl standard culture media supplemented with 1% or 10% CSS, with or without 0.1 nM DHT. The EV suspensions were then added to the LNCaP cells (total volume/well = 200 µl) and co-cultured together for 96 hours. LNCaP cells treated with an equal volume of PBS served as controls. Phase-contrast images of each well were collected every two hours for a total of four days, and cell confluency was calculated using the IncuCyte™ image analysis software.

2.1.3 Protein and RNA isolation and analysis

2.1.3.1 Western blot analysis

To prepare samples for western blotting, cells were lysed in RIPA buffer (Sigma) supplemented with a mixture of protease and phosphatase inhibitors, then centrifuged at 13,500 × *g* (10 minutes, 4°C) to remove nuclei and cellular debris. Protein content was quantified using the BCA Protein Assay Kit. Equal amounts of protein, or normalised EV preparations (see details above), were mixed with Laemmli sample buffer (4x) containing β-mercaptoethanol (5% v/v)*, heated (10 minutes, 95°C), then separated on a 10% mini PROTEAN® precast gel (BioRad) for 1 hour at 140 V. Separated proteins were transferred to an Immobilin®-FL polyvinylidene difluoride (PVDF) membrane (0.22 µm pore size) for 1 hour at 100 V and 4°C. Subsequently, membranes were blocked in 5% skimmed milk in TBS containing 0.1% Tween 20 (TBST) for at least 1 hour, then incubated with primary antibodies (Table 2.3) diluted in 5% milk in TBST overnight at 4°C. Following incubation, membranes were washed three times in TBST for 5 minutes each, then probed with the appropriate HRP-conjugated secondary antibody (diluted in 5% milk in TBST) for 1 hour. The membranes were washed a further three times in TBST for 15 minutes each, and then incubated with Clarity™ enhanced chemiluminescence (ECL) solution (Bio-Rad) for 5 minutes before being imaged on the ChemiDoc Touch Imaging System. Relative band intensities were quantified using the ImageJ software. Specifically, a region of interest (ROI) was drawn around the largest protein band in each row and used to define the area within which each of the other bands in the same row were then measured. For CD63 and PAT4, both protein bands appeared as diffuse smears, therefore a relatively large boundary was set around the region in which the signal was visible (for PAT this extended from ~50-250 kDa, and ~37-100 kDa for CD63). The density of each band within the given ROI was then calculated and normalised to a loading control.

Antibody	Antigen	Species	Dilution	Company
PRIMARY	CD63	Mouse	1:500	BD Biosciences
	CD81	Mouse	1:500	Santa Cruz
	AR	Rabbit	1:500	Santa Cruz
	p-S6 (S240/244)	Rabbit	1:1000	Cell Signalling
	S6 (pan)	Rabbit	1:1000	Cell Signalling
	GOLGA2	Rabbit	1:500	Abnova
	Rab11	Mouse	1:500	BD Biosciences
	p-Akt (S473)	Rabbit	1:1000	Cell Signalling
	Akt (pan)	Rabbit	1:1000	Cell Signalling
	4E-BP1	Rabbit	1:1000	Cell Signalling
	EEA1	Rabbit	1:500	Cell Signalling
	Flotillin-2	Rabbit	1:500	Cell Signalling
	PSA	Mouse	1:500	Santa Cruz
	Calnexin	Rabbit	1:500	Abcam
	Syntenin-1	Rabbit	1:1000	Abcam
	Tsg101	Rabbit	1:500	Abcam
	p-Rb (Ser780)	Rabbit	1:500	Abcam
	p-Smad1/5 (S463/465)	Rabbit	1:500	Cell Signalling
	α-Tubulin	Mouse	1:1000	Sigma
	Rb	Mouse	1:500	BD Biosciences
	Alix	Mouse	1:500	Abcam
	LAMP-1	Rabbit	1:1000	Cell Signalling
	PAT4	Mouse	1:200	Novus
SECONDARY	HRP-conj anti-rabbit	Donkey	1:5000	Promega
	HRP-conj anti-mouse	Donkey	1:5000	Promega

Table 2.3. List of antibodies used for Western blotting

Gene	Forward sequence (5' to 3')	Reverse sequence (3' to 5')
SLC1A5	GGGCAAAGAGTAAACCCACA	CACCATGGTTCTGGTCTCCT
PSA	GACCAAGTTCATGCTGTGTG	ACTAGGGAGCCATGGAGGAC
ACTIN	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
AR	AAGACCTGCCTGATCTGTGGAG	CCAGATCATCCCTGCTTCAT
BMP-6	CTCGAGAGAGATAAGGACTGAG	CTCGAGACTCGCATGAGATAGT
p21	GGCAGACCAGCATGACAGATT	GCGGATTAGGGCTTCCTCTT
RB	GGAAGCAACCCTCCTAAACC	TTTCTGCTTTTGCATTCGTG
MCM7	ACGTCAGCGTCACTGGTATTT	CTTCCAGGTAGGTTTCTGAGAGT
FANCI	CCACCTTTGGTCTATCAGCTTC	CAACATCCAATAGCTCGTCACC
TMPRSS2	GTGATGGTATTCACGGACTGG	CAGCCCCATTGTTTTCTTGTA
LMNB1	GCTCTTGCTACTGCACTTGGT	GTAAGGCTCTGACAACGATTCTC

Table 2.4. List of primer sequences used for qRT-PCR

2.1.3.2 Quantitative RT–PCR

Total RNA was extracted using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (New Brunswick) and then reverse transcribed into cDNA using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was assessed via quantitative real-time PCR (qRT-PCR) using Power SYBR Green Master Mix (Applied Biosystems) and monitored using a StepOne™ Real-Time PCR system (Applied Biosystems). Each sample was analysed in triplicate and mRNA levels normalised to β-actin. All qRT-PCR primer sequences are listed in Table 2.4.

*When probing for CD63, CD81 or PAT4, β-mercaptoethanol was omitted from the sample preparations

2.1.4 Analysis of BMP-6 treatment on PCa cell behaviour

2.1.4.1 Proliferation assays

NucLight red-expressing LNCaP (3000 cells/well) and PC-3 cells (2500 cells/well) were seeded into 96-well plates in their standard culture medium supplemented with 10% CSS. The next day, cells were treated with 200 ng/ml BMP-6 (R&D Systems) and the plates were placed inside the IncuCyte ZOOM™ for imaging. Phase-contrast and fluorescent images were collected every two hours for a total of four days, and the number of red objects (1/mm^2) were quantified using the IncuCyte™ object counting algorithm. The rate of cell proliferation was calculated using the following formula: (no. red objects at n hour – no. red objects at 0 hour) / (no. red objects at 0 hour) × 100%.

2.1.4.2 Cell growth assays

LNCaP (3000 cells/well), C4-2B (3000 cells/well), DU145 (2500 cells/well) and PC-3 (2500 cells/well) cells were seeded into 96-well plates in 100 µl of their respective culture medium supplemented with 10% CSS. Following overnight incubation, cells were treated with 0, 1, 10, 50, 100 or 200 ng/ml BMP-6 in 100 µl of medium and the plates were placed inside the IncuCyte ZOOM™ for imaging. Phase-contrast images were collected every two hours for a total of four days, and cell confluency was calculated using the IncuCyte™ image analysis software.

2.1.4.3 Co-culture assays

2.1.4.3.1 Direct

To analyse the effects of the bone stroma on prostate cancer cell proliferation *directly*, NucLight Red-expressing LNCaP and PC-3 cells were utilised. For the LNCaP/bone stromal cell co-culture experiments, a total of 6,000 cells per well were seeded into 96 well plates. Specifically, in the case of the LNCaP/HS-5 or LNCaP/HS-27a co-cultures, cells

were mixed in a 1:1 ratio (3,000 cells each), whereas cells were mixed in a 2:1:1 ratio (3,000 LNCaP cells; 1,500 HS-5 cells; 1,500 HS-27a cells) in the LNCaP/HS-5/HS-27a co-culture assays. LNCaP monocultures (6,000 cells per well) were used as controls.

Regarding the PC-3/bone stromal cell co-cultures, a total of 4,000 cells per well were seeded into 96 well plates. Again, cells were mixed in a 1:1 ratio (2,000 cells each) in the PC-3/HS-5 or PC-3/HS-27a co-cultures, and a 2:1:1 ratio (2,000 PC-3 cells; 1,000 HS-5 cells; 1,000 HS-27a cells) in the PC-3/HS-5/HS-27a co-cultures. Monocultures of PC-3 cells (4,000 cells per well) were used as controls. All cells were plated in RPMI-1640 medium supplemented with 10% CSS and immediately placed inside the IncuCyte ZOOM™ for imaging. Phase-contrast and fluorescent images were collected every two hours for a total of four days, and the number of red objects (1/mm^2) were quantified using the IncuCyte™ object counting algorithm.

2.1.4.3.2 Indirect

PCa cells were plated at the following densities; 1.8 x 10^5 LNCaP cells per well, 1.5 x 10^5 C4-2B cells per well and 1 x 10^5 PC-3 cells per well in 12 well plates. HS27a and HS-5 stromal cells were mixed in a 1:1 ratio (7.5 x 10^4 each; 1.5 x 10^5 total cells) and seeded into transwell inserts [0.4 µm] (NUNC). All cells were plated in RPMI-1640 medium supplemented with 10% CSS and allowed to attach overnight in separate plates before co-culturing. The next day, the inserts containing the HS27a/HS-5 cell mixtures were added to the wells containing the prostate cancer cells, and those wells without inserts were used as controls. Fresh media was added, and the plates were then placed inside the IncuCyte ZOOM™ for imaging. Phase-contrast images were collected every two hours for a total of four days, and cell confluency was calculated using the IncuCyte™ image analysis software.

2.1.4.4 Luciferase assay

A total of 2 x 10^5 LNCaP, C4-2B and HEK293T cells per well were plated into 12 well plates. The next day, cells were co-transfected with 1 µg each ARE-Luc and Renilla-Luc (both kind gifts from Dr Carlo Rinaldi). A CD63-GFP plasmid was also transfected separately, but alongside the luciferase constructs, to monitor transfection efficiency. Twenty-four hours post-transfection, cells were split and seeded at a density of 3000 cells per well into 96 well plates in media supplemented with 10% CSS. The next day, cells were treated with vehicle (methanol/BSA), BMP-6 (200 ng/ml), DHT (10 nM) or DHT and BMP-6 together for 24 or 48 hours (five wells/condition). Once these time points had elapsed, cells were lysed, and the luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega), according to manufacturer's instructions. Firefly luciferase activity was normalised to the Renilla luciferase signal.

2.1.4.5 Immunofluorescence

LNCaP (2 x 10^5 cells/well) and C4-2B (1.8 x 10^5 cells/well) cells were seeded onto sterile coverslips in 12-well plates. The next day, the culture medium was changed to RPMI-1640 supplemented with 10% CSS and cells were treated with BMP-6 (200 ng/ml). After 24 hours, the cells were washed briefly with PBS and fixed onto their coverslips with 4% (w/v) PFA for 15 minutes at room temperature. The coverslips were washed twice more in PBS before the cells were subjected to permeabilisation with 0.05% TritonX-100/PBS for 30 seconds. Following a further three additional washes in PBS, the cells were incubated with anti-AR antibody (Santa Cruz) diluted in 5% donkey serum/PBS in a humid chamber at 4°C, overnight. The following day, the coverslips were washed three times in PBS and then incubated with Alexa Fluor 488 anti-rabbit secondary antibody (Jackson Lab) diluted in 5% donkey serum/PBS for 1 hour at room temperature, shielded from light. Finally, after three further PBS washes, the coverslips were mounted onto glass microscope slides (Fischer Scientific) with DAPI-containing mounting medium (VectorShield; Vector Laboratories) and

sealed with clear nail polish. Slides were stored at 4°C before imaging. Confocal images were acquired in collaboration with Dr Irina Stefana (Wellcome Trust Centre for Human Genetics, University of Oxford) and Aashika Sekar (Wilson Lab, DPAG, University of Oxford) using a Zeiss LSM 510 Meta laser scanning confocal microscope equipped with a 63×, NA 1.4 Plan APO oil DIC objective (Carl Zeiss) and operated using LSM 510 Meta software. All antibodies used are listed in Table 2.2.

2.1.5 Statistical analysis

Unless otherwise stated, data are presented as the mean ± SD. A two-tailed Student's t-test was used to compare differences between subgroups. A statistically significant difference was defined as $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$.

CHAPTER 3

RESULTS

3. The role of BMP-6 signalling in the regulation of PCa cell growth

3.1. Introduction

3.1.1 Function and expression of BMPs in PCa

A number of BMP ligands are expressed in the normal prostate, including BMP-2, -3, -4, -6 and -7 (Bentley et al., 1992), where they serve to regulate the development and functioning of the gland (Lamm *et al*, 2001; Settle *et al.,* 2001). Yet, altered expression of certain BMPs correlates with PCa progression, indicating that these ligands may also play a role in the pathogenesis of this disease.

Amongst the BMPs studied, BMP-6 and BMP-7 are two family members most commonly associated with PCa progression and the development of skeletal metastases (Autzen et al., 1998; Morrissey et al., 2010). Despite this, the expression patterns of these two BMPs are different. For instance, BMP-7 expression fluctuates greatly with PCa progression. Indeed, many have reported that BMP-7, whilst detectable in normal prostate tissue, is notably decreased or altogether absent in samples derived from patients with advanced disease (Harris et al., 1994; Thomas et al., 2001; Horvath et al., 2004; Masuda et al., 2004; Spanjol et al., 2010). However, elevated BMP-7 has also been observed in metastatic bone lesions (Thomas et al., 2001; Masuda et al., 2003; Morrissey et al., 2010). Considering BMP-7 expression is driven by androgens (Paralkar et al*.,* 1998), this marked change in expression is believed to reflect the evolution of tumour cells from an androgen-dependent to an androgen-independent phenotype.

Conversely, expression of BMP-6 reportedly increases as PCa progresses. For example, elevated levels of BMP-6 have been observed in aggressive primary tumours and tumours that have metastasised to bone (Bentley et al., 1992; Barnes et al., 1995; Hamdy et al.,

1997; Autzen et al., 1998). Interestingly, the bone stroma has recently been shown to induce BMP-6 expression in PCa cells *in vitro* (Lee et al., 2016), perhaps explaining why BMP-6 is often elevated in lesions derived from skeletal metastases, yet undetectable in samples obtained from patients with localised disease.

In terms of function, the most commonly reported effect of BMP-6 and BMP-7 on PCa cell growth is inhibition (Haudenschild et al., 2004; Kim et al, 2004; Miyazaki et al., 2004; Morrissey et al, 2010; Kobayashi et al., 2011). However, this effect has also been noted to vary with cell type. Indeed, more aggressive PCa cell lines are typically resistant to BMP-induced growth-inhibition (Dai et al., 2005; Darby et al., 2008; Morrissey et al., 2010; Freeley et al., 2006). Confusingly, however, contradictory findings have been reported by different groups investigating the effects of the same BMP ligands on the growth of the same PCa cell lines. For instance, treatment with either BMP-6 or BMP-7 has been noted to inhibit and promote LNCaP cell proliferation (Kim et al., 2004, Haudenschild et al., 2004; Lee et al., 2014; Darby et al., 2008). Similarly, unchanged and decreased growth have both been reported in PC-3 cells and PC-3M cells (a highly metastatic PC-3- derived cell line) treated with BMP-6 and BMP-7, respectively (Kim et al., 2004; Darby et al., 2008; Miyazaki et al., 2004; Morrissey et al., 2010). Explanations for such conflicting results centre upon the reported differences in cell culture conditions between the studies. For example, the percentage serum and the level of androgens or anti-androgens in the media, as well as the concentration of BMP ligands, are all factors that have been noted to differ.

In addition to the results lacking consensus, the general finding that BMP-6 and BMP-7 inhibit PCa cell growth is also confusing given our knowledge of their expression profiles. In terms of BMP-7, this effect is perhaps easier to understand considering the expression of BMP-7 typically decreases as PCa progresses. However, the anti-proliferative effect reported for BMP-6 seems to contradict the positive correlation which has been established between elevated BMP-6 levels and the development of aggressive PCa. However, as

discussed in the introduction (section 1.3.2.4), several studies have hinted that enhanced signalling via the BMP-6 pathway may be linked to the development of CRPC. Hence, in this chapter, I focus on unpicking the mechanisms by which BMP-6 regulates PCa cell growth in order to determine the clinical potential this ligand may hold as a therapeutic target.

3.1.5 Aims and objectives

i. Establish conditions to examine the effects of BMP-6 on PCa cell growth
ii. Characterise the effects of BMP-6 on the growth of PCa cell lines
iii. Investigate the mechanisms underlying the effects of BMP-6 on PCa cell growth

3.2 Results

3.2.1 Establishing conditions to examine the effects of BMP-6 on PCa cell growth

Given the lack of consensus concerning the effect of BMP-6 on PCa cell growth, and the disparities in cell culture settings between the studies that have previously explored this topic, I decided to conduct a screen to examine the effect of BMP-6 on PCa cell proliferation in a range of different culture conditions. Here, the main aim was to develop a comprehensive understanding of the growth-regulatory functions of BMP-6, and to establish a set of conditions to use for additional studies to further explore the role of this ligand in PCa progression. LNCaP cells were used for the screen as they represent a commonly used PCa cell line studied extensively in the context of BMP signalling. Before beginning the screen, cells were first transduced with lentivirus encoding a nuclear-localised red fluorescent protein so that changes in cell number could be easily monitored. Following puromycin selection, cells were cultured in a variety of conditions, including media supplemented with 1% or 10% CSS, alone, or with DHT (0.1 nM), alone, or in combination with bicalutamide (10 μM). In addition, cultures were treated with a range of BMP-6 concentrations (0, 1, 10, 50 100, 200 ng/ml) and proliferation was monitored in real-time using the IncuCyte ZOOM™ live-cell imaging system, over 96 hours (Figure 3.1).

Overall, the general trend resulting from the screen was that stimulation of LNCaP cells with increasing concentrations of BMP-6 inhibited cell proliferation, with some exceptions. For instance, there was also evidence that BMP-6 could elicit a biphasic growth response in certain conditions. Several of the assays conducted in low serum (i.e. 1% CSS) showed that weaker concentrations of BMP-6 (1 and/or 10 ng/ml) positively affected cell proliferation (Figure 3.1A). Since this effect was largely absent after equivalent treatments in cells cultured in higher serum conditions (Figure 3.1B), it is possible that additional factors present in serum could have hindered the function of BMP-6, confounding the results. For

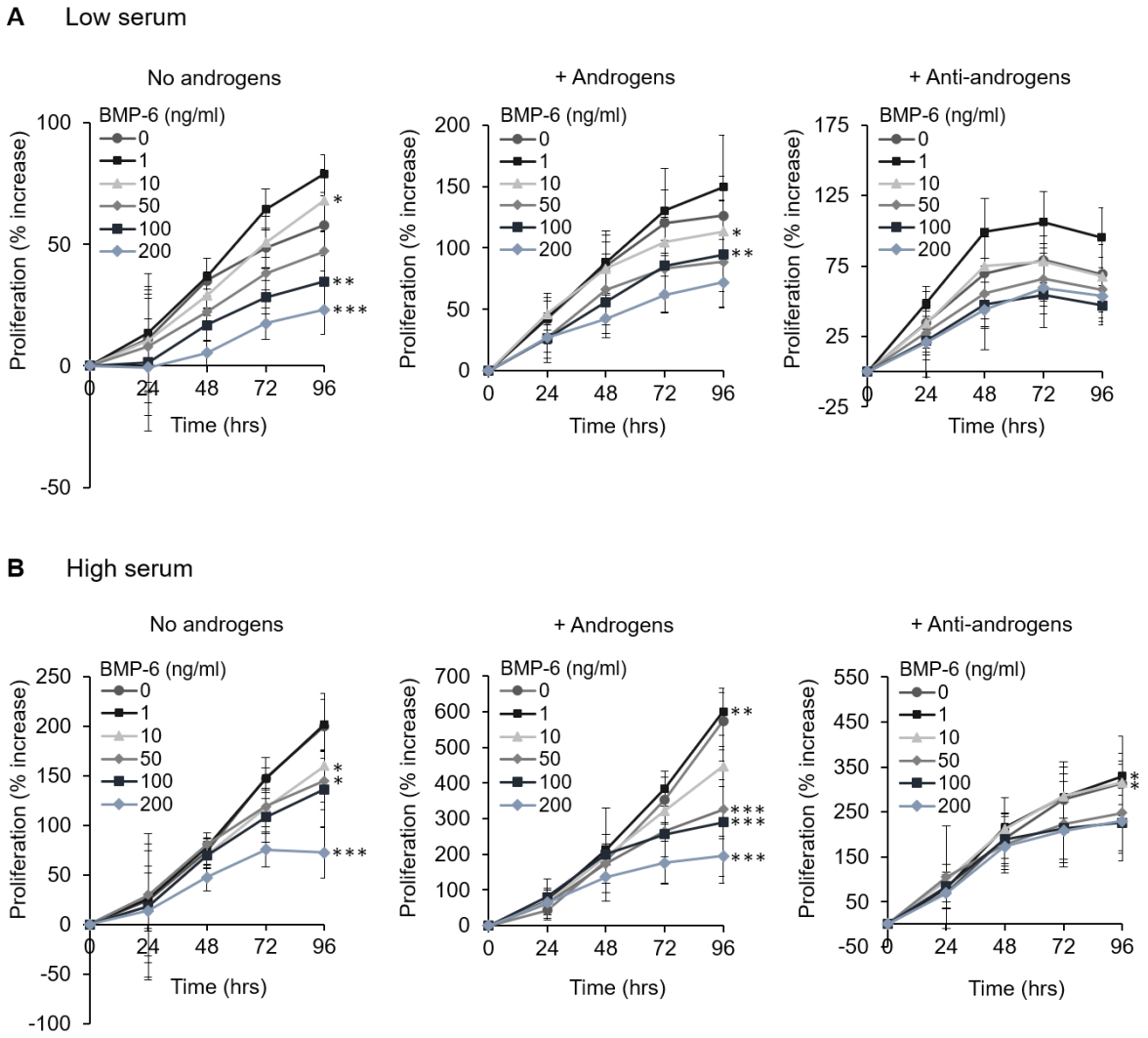


Figure 3.1. BMP-6 inhibits LNCaP cell proliferation in a wide range of culture conditions. Increasing concentrations of recombinant BMP-6 (0, 1, 10, 50 100, 200 ng/ml) was added to NucLight Red-expressing LNCaP cell cultured in either low (1% CSS, Fig **1A**) or high (10% CSS; Fig **1B**) serum conditions, treated with vehicle (methanol/BSA; A and B, **left** panels), DHT (10 nM; A and B, **middle** panels) or DHT plus bicalutamide (10 μM; A and B, **right** panels) for 96 hours. Red fluorescent images of the cells were collected using the IncuCyte ZOOM™ and the number of red objects were quantified using the IncuCyte™ object counting algorithm. Cell proliferation is represented as the fold change in red objects (1/mm^2) between 0 and 96 hours. Results are mean ±S.D of three independent experiments (8 replicates/condition). *P < 0.05, **P < 0.01 or ***P < 0.001 vs. control at 96 hours.

instance, serum-derived BMP-6 itself, or BMP-6 inhibitors (e.g. Noggin) even, could both explain the differential effects observed between the two conditions. Indeed, an important rationale for testing the function of BMP-6 in low serum conditions was to avoid masking specific effects: serum-starved cells are more sensitive to both mitogenic and anti-mitogenic

signals. This may also explain why, in serum-starved settings, the effects of BMP-6 on LNCaP cell proliferation were evident almost immediately yet did not appear until ~24-48 hours in serum-rich conditions, perhaps once serum levels had begun to dissipate (Figure 3.1A versus 3.1B, left panels). Thus, lower serum levels may enable subtler differences in the effects of BMP-6 to be detected. However, deciding whether to use serum-starved conditions in future experiments raised other important issues. For example, elevated levels of cellular stress may hinder the analysis of a BMP-6-induced stress response. I also reasoned that it would be preferable to deviate as little as possible from 'normal' culture conditions unless necessary to observe a specific phenotype. Considering growth inhibition was the most consistent and convincing phenotype stimulated by BMP-6 in LNCaP cells, and that this response was similar in both serum conditions, I decided to culture cells in medium supplemented with 10% serum (as standard) in all further experiments.

In addition to serum levels, I reasoned that another important factor to consider when examining the effects of BMP signalling in the context of PCa was the influence of androgens. As outlined previously, BMP-6 is commonly elevated in men with late-stage, bone metastasised PCa. Hence, it is reasonable to assume that such patients would have received, or currently be undergoing, hormone-deprivation therapy and therefore be experiencing low levels of circulating androgens. Consequently, the effects of BMP-6 on LNCaP cell proliferation was also examined in the presence of DHT, with or without addition of the AR antagonist bicalutamide (Figure 3.1A and 3.1B, middle and right panels).

Overall, compared to the effects in androgen-deprived conditions, culturing LNCaP cells with DHT did not appear to affect the cells response to BMP-6. For instance, in serum-rich conditions, regardless of whether androgens were present in the medium or not, treatment with BMP-6 at concentrations of 10 ng/ml and above inhibited LNCaP cell proliferation (Figure 3.1B, left versus middle panel). Intriguingly however, blocking AR signalling with bicalutamide treatment strongly diminished the growth-inhibitory effects of BMP-6,

suggesting that AR activity may be necessary to elicit such a response (Figure 3.1B, middle versus right panel). The result was less clear in serum-depleted conditions although, broadly, the same general trend was observed (Figure 3.1A). Whilst these findings indicated that AR signalling likely affected the function of BMP-6, I reasoned that a sensible strategy was to first characterise the effects of BMP-6 alone before considering any interplay with additional factors. To this end, cell culture experiments presented in the rest of this chapter are conducted in a medium supplemented with 10% CSS to mimic androgen-deprived conditions unless otherwise stated.

Ultimately, the screen was successful in fulfilling the proposed aims. Namely, providing greater insight into the growth-regulatory functions of BMP-6, as well as helping to highlight the culturing conditions to use in further studies.

3.2.2 Characterising the effects of BMP-6 on the growth of a panel of PCa cell lines

To examine the function of BMP-6 in the context of late-stage PCa, a panel of PCa cell lines derived from patients during the advanced stages of the disease was utilised. A total of four, morphologically and phenotypically distinct cell lines were chosen to represent the heterogeneity commonly observed in metastatic PCa. For instance, LNCaP is a luminal-epithelial cell line developed from a prostate adenocarcinoma lesion derived from lymph node metastases; C4-2B cells are a subline of LNCaP that have been generated through long-term androgen-deprivation and propagation of metastatic lesions in mice; DU-145 cells are derived from a brain-metastasised tumour of prostate adenocarcinoma origin; and PC-3 cells are tumour cells isolated from a vertebral metastatic prostate lesion (see Table 2.1 for further details). Strategically, by screening the effects of BMP-6 in a panel of different PCa cell lines, I hoped that further clues as to the role BMP-6 plays in PCa progression would be revealed. Experimentally, cells were plated in their standard culture medium supplemented with 10% CSS, then treated with a range of BMP-6 concentrations for a period of 96 hours. As all cell lines had not been transduced with nuclear RFP, cell

confluency was measured using the IncuCyte ZOOM™ to examine changes in cell growth instead.

The effect of BMP-6 varied considerably depending on the cell line tested. For example, in LNCaP cells, BMP-6 elicited an inhibitory effect on LNCaP cell confluency at concentrations of 50 ng/ml and above (Figure 3.2A, left panel). This inhibitory effect was also observed for LNCaP cell proliferation and to a similar degree, with the highest BMP-6 concentration reducing cell confluency by ~50% at 96 hours versus a ~60% decrease in proliferation at the same time point (compare Figure 3.2A with Figure 3.3A). Conversely, BMP-6 stimulated a positive response in the growth of PC-3 cells (Figure 3.2B, left panel). Indeed, even with the lowest concentration of ligand tested (1 ng/ml) cell confluency was moderately, but significantly, increased; whereas the highest concentration of BMP-6 (200 ng/ml) produced a ~50% increase in PC-3 cell growth at 96 hours. This effect, however, did not translate into an increase in cell proliferation (Figure 3.3B), suggesting that the changes in cell confluency may have reflected alterations in PC-3 cell morphology instead. In contrast to both of these results, BMP-6 had no discernible effect on the growth of either C4-2B or DU-145 cells (Figure 3.2C and 3.2D, left panels). Hence, the effect of BMP-6 on growth seemed to be somewhat cell type-specific. To assess whether this was due to differences in the expression of the BMP signalling machinery (e.g. cell surface receptors) between the cells, downstream BMP activity was examined in each line following BMP-6 stimulation (Figures 3.2A, 3.2B, 3.2C and 3.2D, right panels).

As previously detailed, after binding to their cognate cell surface receptors, BMP ligands catalyse a downstream signalling cascade characterised by phosphorylation of certain Smad proteins. As BMP-6 is believed to principally signal through Smad1, Smad5 and Smad8, the phosphorylation status of these proteins was examined in all four PCa cell lines following treatment with BMP-6 (200 ng/ml) or vehicle for three hours. Western blot analysis revealed a significant increase in the phosphorylation of Smad1/5 in LNCaP, C4-2B and

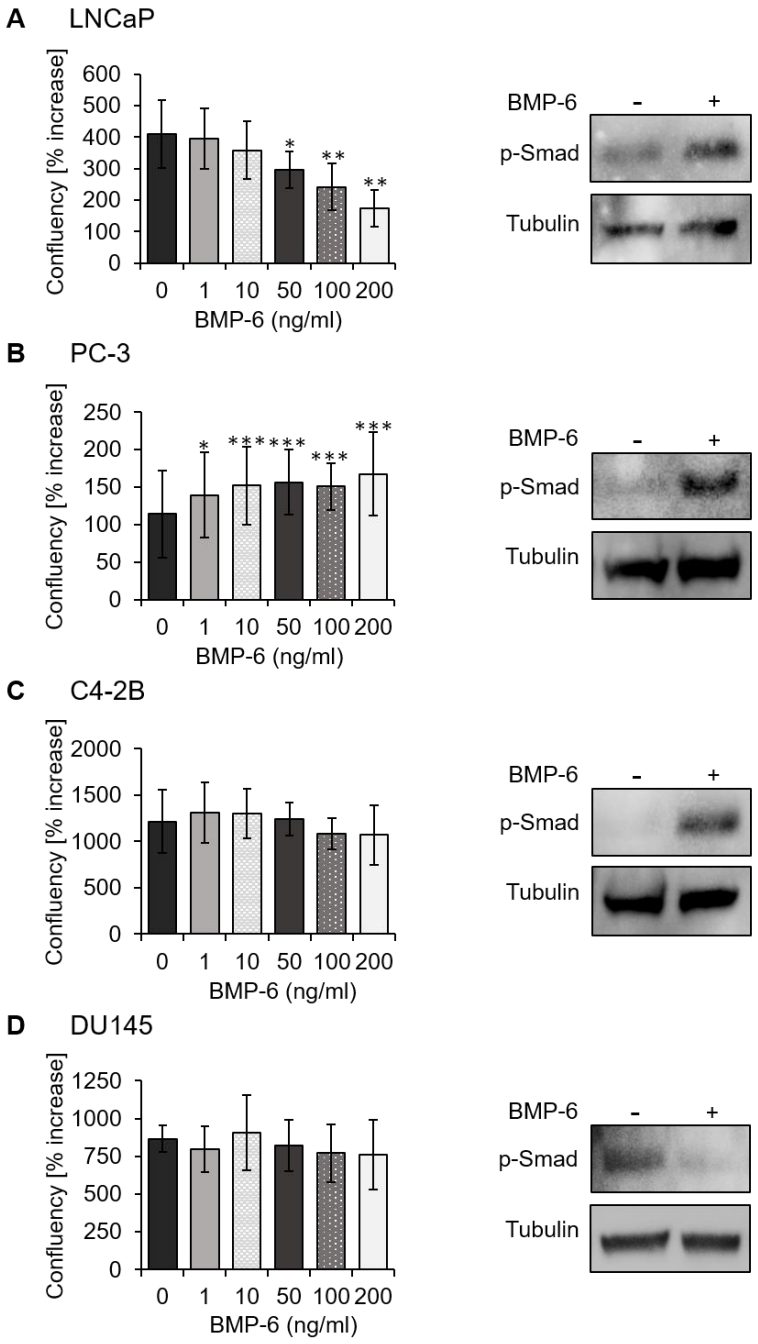


Figure 3.2. BMP-6 can differently affect PCa cell growth. (A-D, **left** panel) Increasing amounts of recombinant BMP-6 (0, 1, 10, 50 100, 200 ng/ml) was added to LNCaP, PC-3, C4-2B and DU145 cell cultures for 96 hours. Phase-contrast images were collected using the IncuCyte ZOOM™ and cell confluency was calculated using the IncuCyte™ software. Cell growth is represented as the fold change in percentage confluency between 0 and 96 hrs. Results are mean ±S.D of three independent experiments (8 replicates/condition). **P* < 0.05, ***P* < 0.01 or ****P* < 0.001 vs. control at 96 hours. (A-D, **right** panel) Cells were cultured with or without BMP-6 (200 ng/ml) for 1 hour and the downstream effects on p-Smad (1/5) examined.

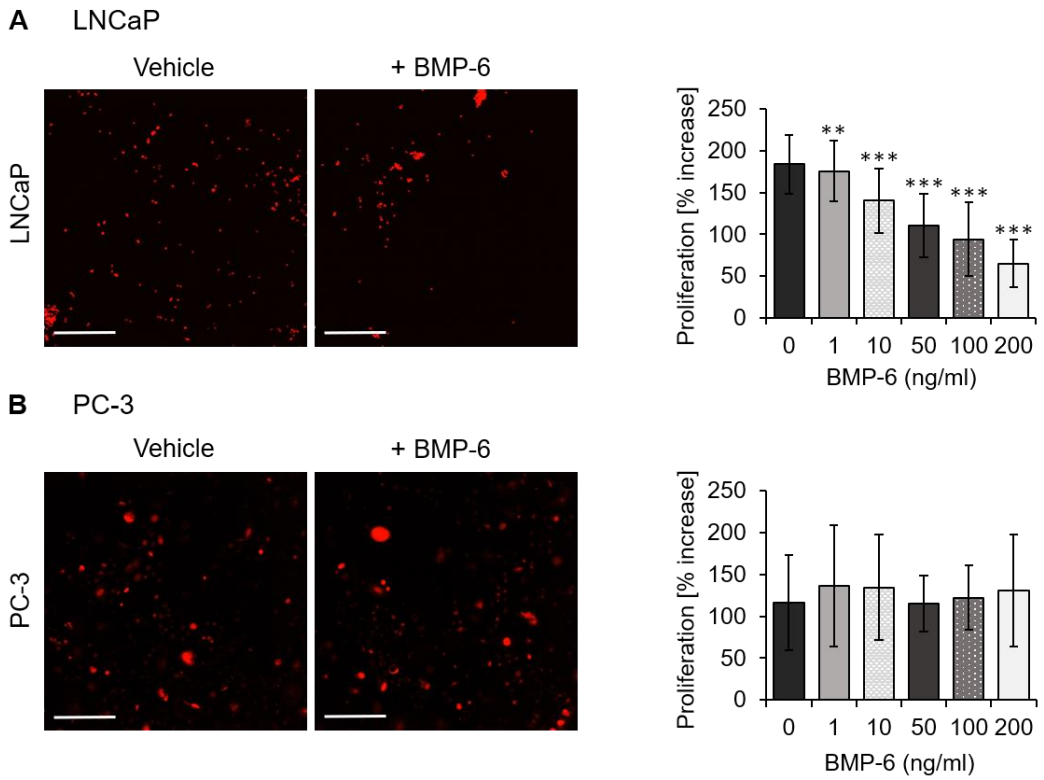


Figure 3.3. BMP-6 inhibits LNCaP, but not PC-3, cell proliferation. (A & B, **right** panels) Increasing concentrations of recombinant BMP-6 (0, 1, 10, 50 100, 200 ng/ml) was added to NucLight Red-expressing LNCaP and PC-3 cells for 96 hours. Red fluorescent images of the cells were collected using the IncuCyte ZOOM™ and the number of red objects were quantified using the IncuCyte™ object counting algorithm. Cell proliferation is represented as the fold change in red objects (1/mm^2) between 0 and 96 hours. (A & B, **left** panel) Representative images of cells treated with and without BMP-6 (200 ng/ml) at 96 hours are shown. Results are mean ±S.D of three independent experiments (8 replicates/condition). ***P* < 0.01 or ****P* < 0.001 vs. control at 96 hours. Scale bar = 300 µm.

PC-3 cells upon BMP-6 addition, signifying that the Smad signalling pathway was functional in these cells and utilised by BMP-6 for signal transduction (Figures 3.2A, 3.2B and 3.2C, right panels). Conversely, stimulation with BMP-6 inhibited Smad1/5 phosphorylation in DU-145 cells (Figure 3.2D, right panel), suggesting that alternative regulatory mechanisms may be activated in these cells following BMP stimulation - a negative feedback loop perhaps. Hence, the absence of a conventional BMP signalling response could perhaps explain why BMP-6 had no observable effect on DU-145 cell growth. Nonetheless, the same rationale cannot be used to explain the differential growth responses in the other cell lines examined.

Therefore, further analysis of BMP-6 function was continued in the LNCaP, PC-3 and C4-2B cell lines.

3.2.3 Investigating how the bone microenvironment affects PCa cell growth

It has been widely reported that, in PCa, BMP-6 is commonly elevated in patients with established skeletal metastases. Moreover, additional studies have demonstrated that crosstalk between the bone environment and PCa cells can stimulate the secretion of BMP-6. Hence, to try and understand how BMP-6 regulates PCa cell growth *in vivo*, bone stromal cells were used in the following studies instead of exogenous BMP-6 to better emulate the environment in which the ligand typically functions. Specifically, HS-5 and HS-27a, two human BM-derived stromal cell lines were utilised for this work as each represents one of the two major stromal cell phenotypes found in the BM (Graf et al, 2002). Experimentally, each cell line was directly co-cultured (independently and together) with equal numbers of NucLight-labelled LNCaP and PC-3 cells for 96 hours. Labelling the PCa cell lines with RFP enabled me to differentiate between these cells and the bone stromal cell lines, and easily monitor cell proliferation using the IncuCyte ZOOM™.

Interestingly, co-culturing LNCaP cells with bone stromal cells had a similarly inhibitory effect on proliferation to BMP-6 (Figure 3.4A). Growth was significantly reduced (>80%) at 96 hours in every co-culture condition tested relative to the LNCaP monoculture control, but least so when co-cultured with HS-27a cells. Furthermore, there was no discernible difference between the growth of LNCaP cells cultured with HS-5 cells or with a mixture of HS-5/HS-27a cells; both conditions resulted in a ~112% decrease in cell proliferation at 96 hours, compared to the LNCaP monoculture control (Figure 3.4A, lower panel). The fact that co-culturing LNCaP cells with HS-5 and HS-27a together produced a different effect on proliferation to that expected (i.e. an intermediary between co-culturing each individually), suggested to me that a more complex interplay of factors was occurring.

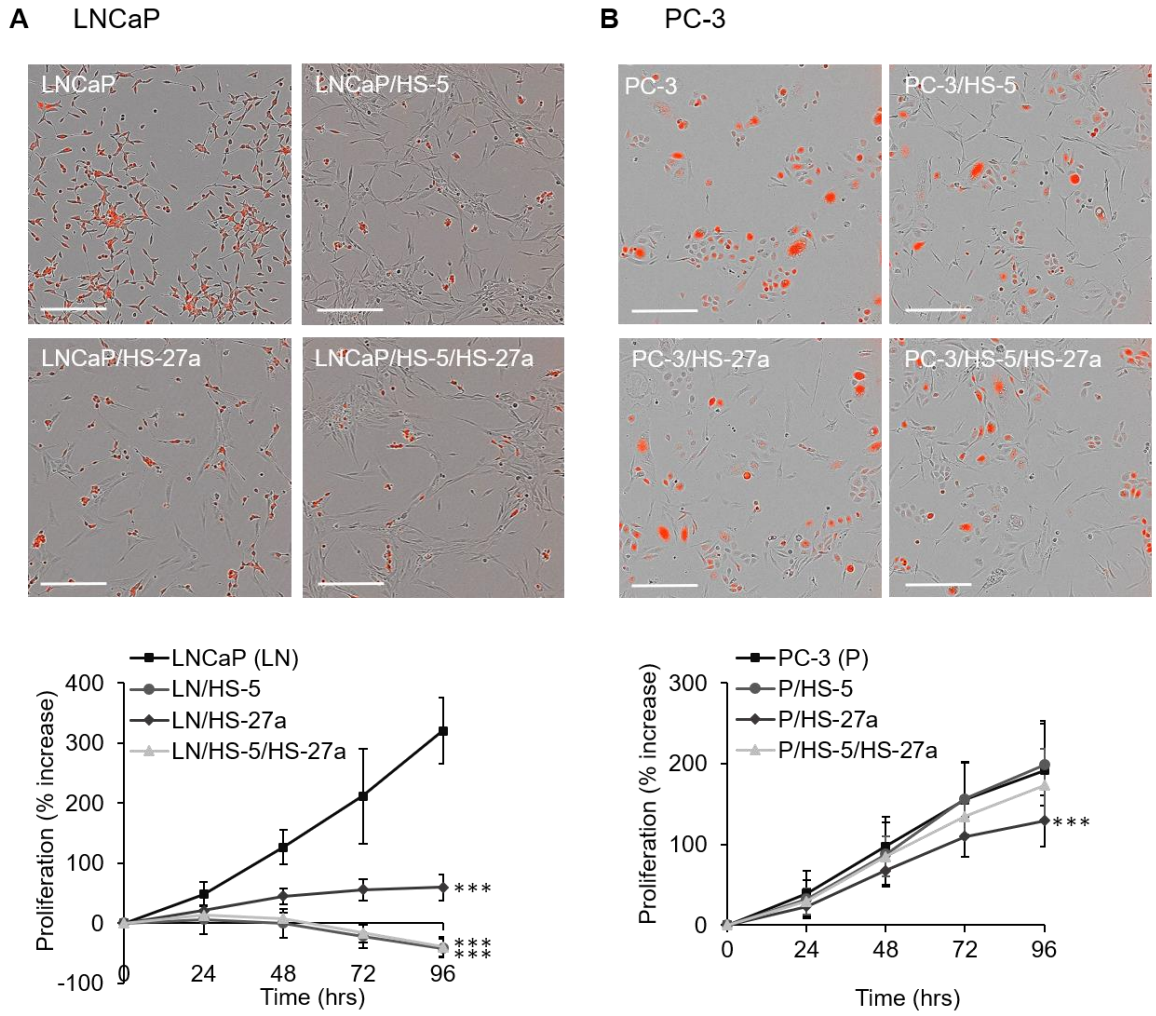


Figure 3.4. Direct co-culture with bone marrow stromal cells mimics the effect of BMP-6 on LNCaP and PC-3 cell proliferation. (A & B, **lower** panels) NucLight Red-expressing LNCaP and PC-3 cells were directly co-cultured with equal numbers of HS-5 and HS-27a cells together for 96 hours. Phase-contrast and fluorescent images of the cells were collected every two hours using the IncuCyte ZOOM™ and the number of red objects were quantified using the IncuCyte™ object counting algorithm. Cell proliferation is represented as the fold change in red objects (1/mm^2) between 0 and 96 hours. (A & B, **upper** panels) Representative images of LNCaP and PC-3 mono- and co-cultures with bone marrow stromal cells are shown at 96 hours. Results are mean ±S.D of three independent experiments (8 replicates/condition). ****P* < 0.001 vs. control at 96 hours.

Another possible explanation could have been that LNCaP cell proliferation reached a point of saturation upon co-culture with HS-5 cells, and that this level was still reached with half as many HS-5 cells and the presence of HS-27a cells (i.e. the setup using the mixed stromal cell co-culture).

Likewise, co-culturing PC-3 cells with bone stromal cells had a similar effect to BMP-6 on proliferation. That is, no effect was observed - with one exception (Figure 3.4B). When co-cultured with HS-27a cells, the growth of PC-3 cells was reduced by ~40% at 96 hours compared to the PC-3 monoculture control (Figure 3.4B, lower panel). However, as was observed with the LNCaP co-culture, the effect of HS-27a cells on PC-3 cell proliferation was effectively lost when the two bone stromal cell lines were cultured together. Again, hinting that the stromal cells may function differently in alternative settings, and that the effect of both together should not be viewed as the sum of either alone. In addition, the fact that the co-culture assay did not produce a similar inhibitory response in PC-3 cells to that seen in LNCaP cells, further suggested that the suppressive effect of the bone stroma on LNCaP cell growth was specific and not a general consequence of the co-culture.

Overall, it appeared as though the bone stroma was impacting LNCaP and PC-3 cell proliferation in a similar manner to BMP-6, with certain disparities. For instance, whilst BMP-6 treatment reduced LNCaP cell growth, the degree of inhibition was far greater when the cells were co-cultured with the bone stromal cells. Specifically, there was a ~60% decrease in LNCaP cell proliferation following treatment with BMP-6 (200 ng/ml), and a ~112% decrease upon co-culture with HS-5/HS-27a cells at 96 hours. Similarly, stimulation with BMP-6 had no effect on PC-3 cell growth. Whilst this finding was largely consistent with the effects observed when PC-3 cells were co-cultured with HS-5 cells or HS-5/HS-27a cells together, incubation with HS-27a cells alone inhibited PC-3 cell proliferation. One possibility I considered was that other factors secreted from the bone stoma may have altered the activity of BMP-6. However, it was unclear at this point whether BMP-6 was even present in these co-culture settings, never mind responsible for the effects on growth. Thus, additional studies were carried out to examine the involvement of BMP-6.

Given that BMP-6 is a secreted growth factor, I reasoned that: if BMP-6 was involved in mediating the effects of the bone stroma on PCa cell growth, then these effects would

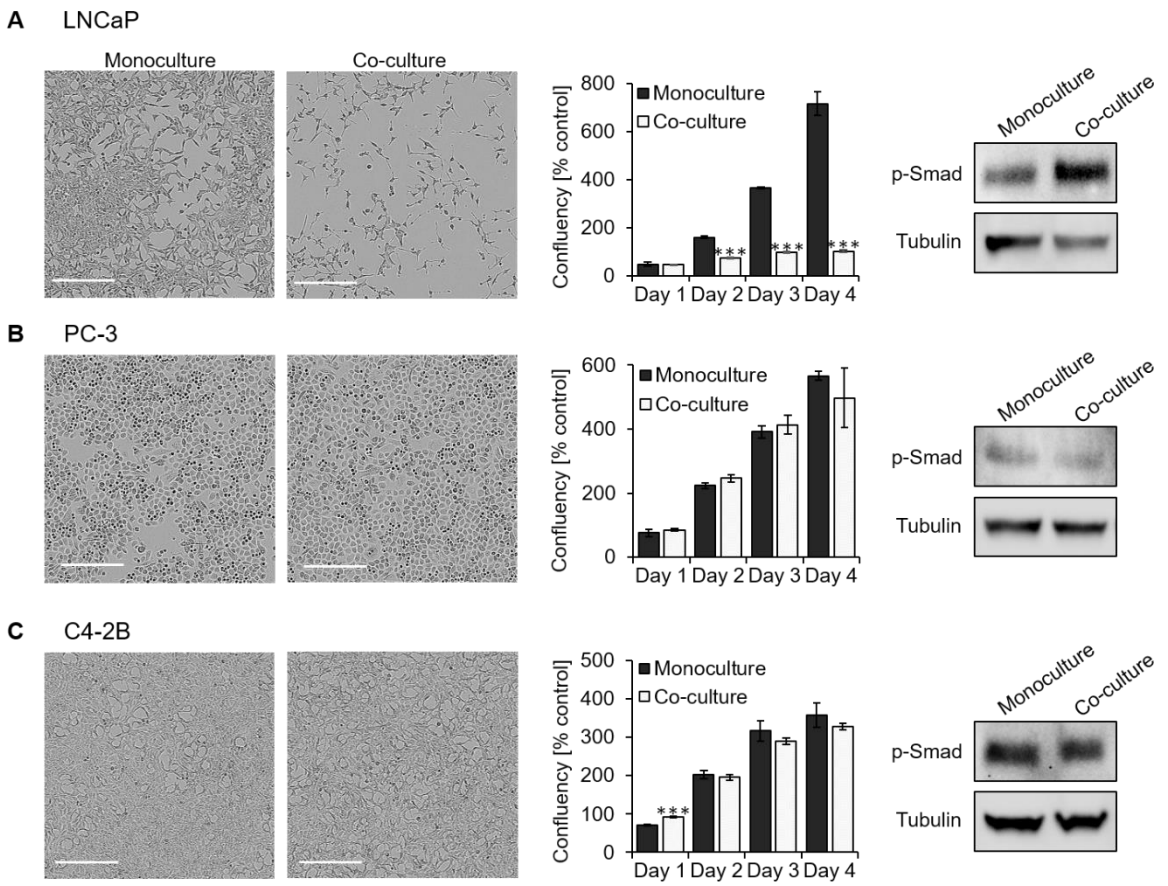


Figure 3.5. Indirect co-culture with bone marrow stromal cells mimics the effect of BMP-6 on the growth of LNCaP, PC-3 and C4-2B cells. (A-C, **middle** panels) LNCaP, PC-3 and C4-2B cells were each indirectly co-cultured with equal numbers of HS-5 and HS-27a cells together for 96 hours. Phase-contrast images were collected every two hours using the IncuCyte ZOOM™ and cell confluency was calculated using the IncuCyte™ software. Cell growth is represented as the fold change in percentage confluency of between 0 and 96 hours. (A-C, **left** panel) Representative images of LNCaP, PC-3 and C4-2B mono- and co-cultures with bone marrow stromal cells are shown at 96 hours. (A-C, **right** panel) Cells were cultured with or without HS-5/HS-27a cells for 24 hours and the downstream effects on p-Smad (1/5/8) examined. Results are mean ±S.D of three independent experiments (8 replicates/condition). ****P* < 0.001 vs. control at 96 hours.

persist when the PCa cells and bone stroma cells were no longer in direct contact. To test this, a co-culture transwell system was established to allow cells in two different compartments to exchange soluble factors without forming cell-cell contacts. A mixture of HS-5 and HS-27a cells were co-cultured indirectly with one of the PCa cell lines; LNCaP, PC-3 or C4-2B for 96 hours (Figure 3.5). In this assay, PCa cells were no longer manipulated to express nuclear RFP as the segregated setup meant that PCa cells could

be identified without the need for fluorescence. Consistent with the results observed in both the direct co-culture assays and with BMP-6 treatment, indirect co-culture with bone stromal cells significantly inhibited LNCaP cell growth (>85%) at 96 hours relative to the LNCaP monoculture control (Figure 3.5A, left and middle panels). Again, however, the extent of this inhibition differed from that seen in either of the other two setups. Here, the reduction in growth was greater than when cells were treated with BMP-6 alone (~50%), but less than when they were directly co-cultured with the HS-5/HS-27a stromal cells (~112%). Therefore, I reasoned that the inhibitory response observed in LNCaP cells when co-cultured directly with the bone stroma could be due, in part, to influences other than secreted factors; whereas the response in the indirect co-culture setting must have been driven by secreted factors which may, or may not, have included BMP-6. Nonetheless, it should also be noted that the metrics used to measure cell growth in the direct co-culture assay (number of red objects/mm^2) differed from those employed in the other two settings (% confluency), and therefore direct comparisons cannot be made.

Also consistent with the results observed in the direct co-culture assay were the effects of the indirect co-culture on PC-3 cell growth (Figure 3.5B). Like the response of PC-3 cells when directly cultured together with HS-5 and HS-27a cells, limiting the contact of these cells to their shared culture medium did not affect PC-3 cell growth. This is in contrast, however, to the increase in cell confluency observed following treatment with BMP-6. Hence, if BMP-6 was present in this environment, its influence on PC-3 cell morphology was no longer evident. Cell growth was also unchanged in C4-2B cells following indirect co-culture with the bone stroma (Figure 3.5C) and mirrored the similar lack in response observed in C4-2B cells following treatment with BMP-6. Therefore, overall, it appeared that the bone stroma stimulated the secretion of certain factor(s) into the environment that limited the growth of LNCaP, but not C4-2B or PC-3, cells.

Although there were several consistent observations between the impact of the bone environment on PCa cell growth and the effects of BMP-6, it was still unclear whether BMP-6 was secreted in the co-culture setting and, if it was, whether it could stimulate a downstream response in PCa cells. To explore this, phosphorylation of Smad1/5 was examined in LNCaP, C4-2B and PC-3 monocultures as well as in the PCa cells following co-culture with HS-5/HS-27a cells for 48 hours. Western blot analysis revealed that phosphorylation of Smad1/5 was upregulated in LNCaP, but not C4-2B or PC-3, cells following co-culture with the bone stromal cell lines (Figure 3.5, right panels). Overall, these findings suggested that the BMP signalling pathway was activated in LNCaP cells cultured in the bone environment and associated with inhibition of cell growth. However, questions remained as to the identity of the BMP responsible for this signal as well as the overall contribution of this pathway to the growth-inhibitory response. Furthermore, as BMP signalling was not stimulated in either C4-2B or PC-3 cells, it was difficult to analyse the effects of BMP-6 on cell growth in the context of the bone environment in these cell lines. Therefore, the remainder of the work presented in this chapter focusses on exploring the mechanisms underlying the differential growth response of PCa cells to exogenous BMP-6 treatment.

3.2.4 Confirming differences in androgen sensitivity between the PCa cell lines

One clear difference between the PCa cell lines examined, which could potentially explain the differing effects of BMP-6 on cell growth, is the sensitivity of the cells to androgen stimulation and expression of the AR. Although each cell line has been previously evaluated for both characteristics, I deemed it important to confirm these findings in my own experimental settings. Initially, AR mRNA and protein expression were examined in each cell line via qPCR and western blotting, respectively. Concordant with previous reports, AR mRNA was expressed in both LNCaP and C4-2B cells, at similar levels, yet absent in PC-3 cells (Figure 3.6A). In terms of protein, unsurprisingly, AR was not expressed at the protein level in PC-3 cells (Figure 3.6B) but was expressed in both LNCaP and C4-2B cells

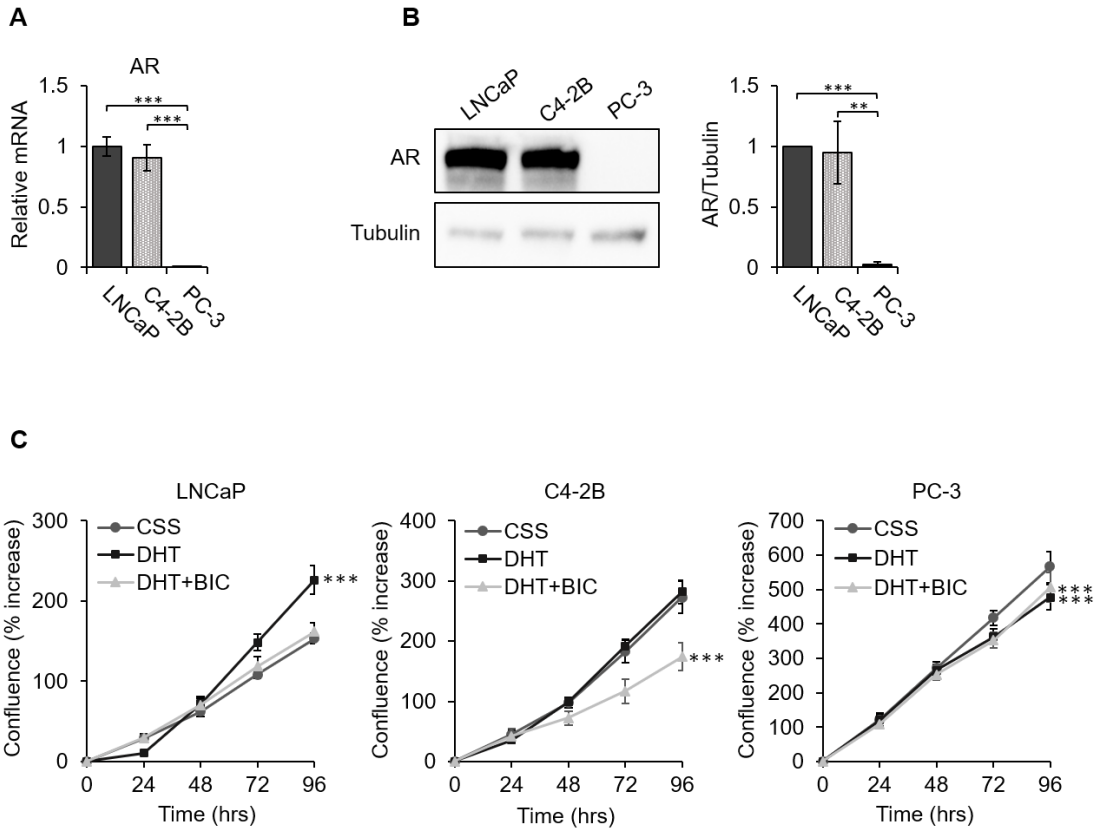


Figure 3.6. Characterisation of AR expression and androgen sensitivity profiles of different PCa cell lines. AR mRNA **(A)** and protein **(B)** levels were examined in LNCaP, C4-2B and PC-3 cells. **(C)** Cells were treated with DHT (0.1 nM) alone or in addition to bicalutamide (10 µM) for 96 hours. Phase-contrast images were collected every two hours using the IncuCyte ZOOM™ and cell confluency was calculated using the IncuCyte™ software. Cell growth is represented as the fold change in percentage confluency of between 0 and 96 hours. (A & B, left panel). Results are mean ±S.D of three experiments (8 replicates/condition). ****P* < 0.001 vs. control at 96 hours.

at similar levels. Functionally, these differences translated into varied responses to androgen and anti-androgen treatment (Figure 3.6C). Specifically, stimulation with DHT (0.1 nM) produced a ~40% increase in LNCaP cell growth after 96 hours; an effect which was abolished following co-treatment with bicalutamide (10 µM; Figure 6C, left panel). This finding was in line with what has been reported for LNCaP cells; that is, that they express functional, but mutated, AR and are therefore sensitive to androgen stimulation and withdrawal. On the other hand, C4-2B cell growth was unaffected by DHT treatment yet inhibited following the addition of anti-androgens (Figure 3.6C, middle panel). Again, the

insensitivity of C4-2B cells to androgen stimulation has been widely described, however the inhibitory response of these cells to bicalutamide treatment indicates that their AR signalling pathway is still functional. Conversely, androgen and anti-androgen treatment had a very subtle inhibitory effect on PC-3 cell growth after 48 hours – a somewhat unexpected result considering the lack of AR expression in these cells (Figure 3.6C, right panel).

Therefore, considering that each cell line exhibited a differential response to both BMP-6 and androgen stimulation, together with my earlier observation that AR signalling may be involved in regulating the growth-inhibitory effects of BMP-6 in LNCaP cells, I decided to explore the role of AR in mediating this response.

3.2.5 Examining mechanisms underlying PCa cell growth

3.2.5.1 Crosstalk with AR signalling

To test the hypothesis that AR signalling is involved in mediating the inhibitory effect of BMP-6 on PCa cell growth, crosstalk between these pathways was examined in LNCaP and C4-2B cells. The decision to exclude PC-3 cells from this analysis reflected the fact that LNCaP and C4-2B cells are both closely related and express the AR yet respond differently to BMP-6 treatment; hence, I hoped they would provide a useful comparison.

Initial work to investigate the effects of BMP-6 on AR signalling involved examining AR expression following BMP-6 treatment. Both cell lines were treated with a concentration of BMP-6 (200 ng/ml) which had been shown earlier to produce the greatest reduction in LNCaP cell growth (and no effect on C4-2B cells). No difference in AR mRNA expression was observed in either cell line upon BMP-6 stimulation at 24 or 48 hours (Figure 3.7A and 3.7B, left panels). Interestingly, however, AR protein was upregulated in both cell lines following BMP-6 treatment (Figure 3.7A and 3.7B, middle and right panels); evidence, perhaps, of BMP-6-induced post-translational modification of the receptor. Comparatively,

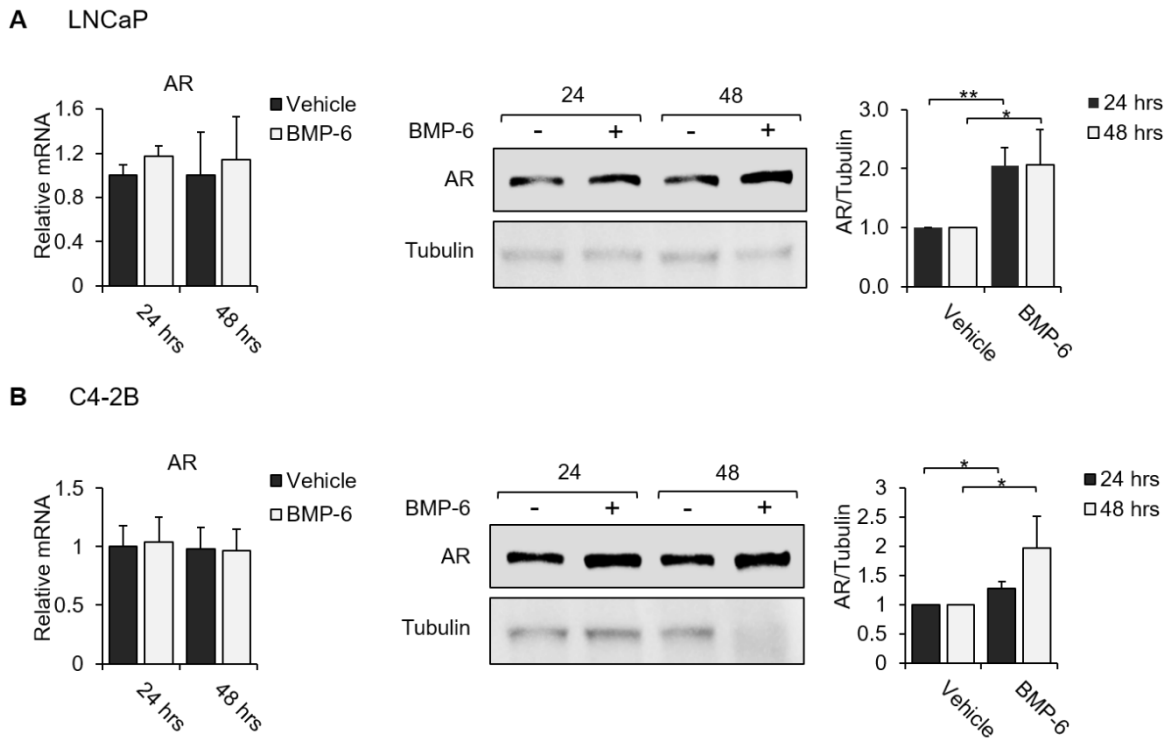


Figure 3.7. BMP-6 increases AR protein levels in LNCaP and C4-2B cells. (**A & B**) LNCaP and C4-2B cells were cultured with BMP-6 (200 ng/ml) for 24 and 48 hours and analysed via qPCR for changes in AR mRNA (**left** panels) and western blotting for changes in AR protein (**right** panels). Results are mean ±S.D of three independent experiments. **P* < 0.05 or ***P* < 0.01 vs. control.

the increase in AR protein was greater in LNCaP cells. Indeed, after 24 hours, there was a ~two-fold increase in receptor levels in these cells versus an increase of around ~1.3 in C4-2B cells. After 48 hours, AR remained at this level in LNCaP cells, but increased to approximately two times that of the control in C4-2B cells. It was therefore evident that BMP-6/AR crosstalk existed in these cells, yet the comparable increase in AR protein begged further questions as to the role such crosstalk played in regulating the differential effects of BMP-6 on LNCaP and C4-2B cell growth.

Furthermore, the increase in AR protein induced by BMP-6 in LNCaP cells was a curious result considering the pro-proliferative function of the receptor in PCa, yet inhibitory action of BMP-6 on LNCaP cell growth. One explanation, which could potentially reconcile these

seemingly contradictory findings, was that BMP-6 was preventing the nuclear translocation of AR, inhibiting the receptor from fulfilling its pro-proliferative functions. Nonetheless, when AR localisation was examined in LNCaP or C4-2B cells with and without BMP-6 stimulation, no difference was observed in either cell line (Figure 3.8). Indeed, immunofluorescence showed that the AR was dispersed between the nucleus and the cytoplasm in LNCaP cells and that this distribution was unchanged following BMP-6 treatment (200 ng/ml; Figure 3.8A). Similarly, in C4-2B cells, the AR appeared enriched within the nucleus in those cells treated with vehicle and remained this way even after the cells were stimulated with BMP-6 (Figure 3.8B). Nonetheless, I reasoned that, whilst AR localisation may be unaffected by BMP-6, it was still possible that the activity of the receptor could have been altered.

To investigate this theory, the effect of BMP-6 on AR-mediated transcription was examined (Figure 3.9). In order to assess changes in AR activity, a synthetic reporter construct encoding a firefly luciferase gene under the control of an AR response element was used, enabling the transcriptional activity of the receptor to be evaluated by monitoring the amount of light emitted. Briefly, cells were transfected with the ARE-Luc construct and then treated with vehicle, BMP-6 (200 ng/ml), DHT (0.1 nM) or DHT plus BMP-6 for 24 or 48 hours before the luciferase signal was measured. Stimulation with DHT was used as a positive control to confirm the assay was functioning correctly, and indeed, increased AR signalling was observed following the addition of androgens in both LNCaP and C4-2B cells after 24 hours (Figure 3.9A and 3.9B, left panels). Conversely, no difference in AR activity was seen in either cell line treated with BMP-6 alone for 24 hours. There was, however, a significant decrease in receptor activity in LNCaP cells following co-treatment with DHT and BMP-6. One possible explanation for these results could be that the basal activity of AR was elevated in the presence of androgens and, therefore, reductions in activity were easier to detect in these conditions.

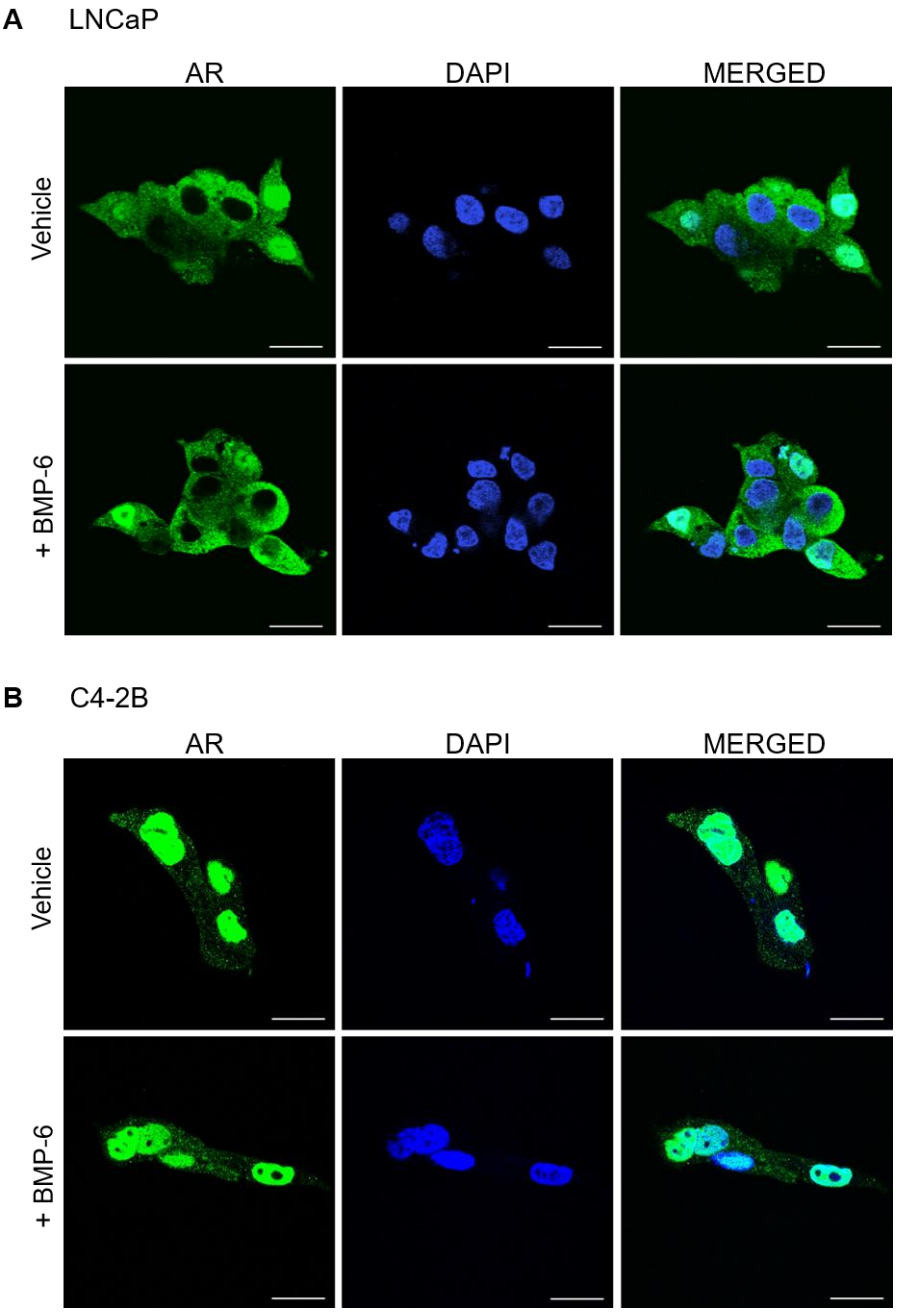


Figure 3.8. BMP-6 does not affect the localisation of AR in LNCaP or C4-2B cells. (**A & B**) Immunohistochemical analysis of AR in LNCaP and C4-2B cells cultured with or without BMP-6 (200 ng/ml) for 24 hours. Representative images shown. Scale bar = 1 µm.

To validate these findings, qPCR was performed to examine the expression of AR-regulated genes in androgen-deprived conditions after 24 hours (Figure 3.9A and 3.9B, right panels). Consistent with the luciferase assay, there was no change in the expression of either PSA or TMPRSS2, two well-characterised transcriptional targets of AR, in C4-2B

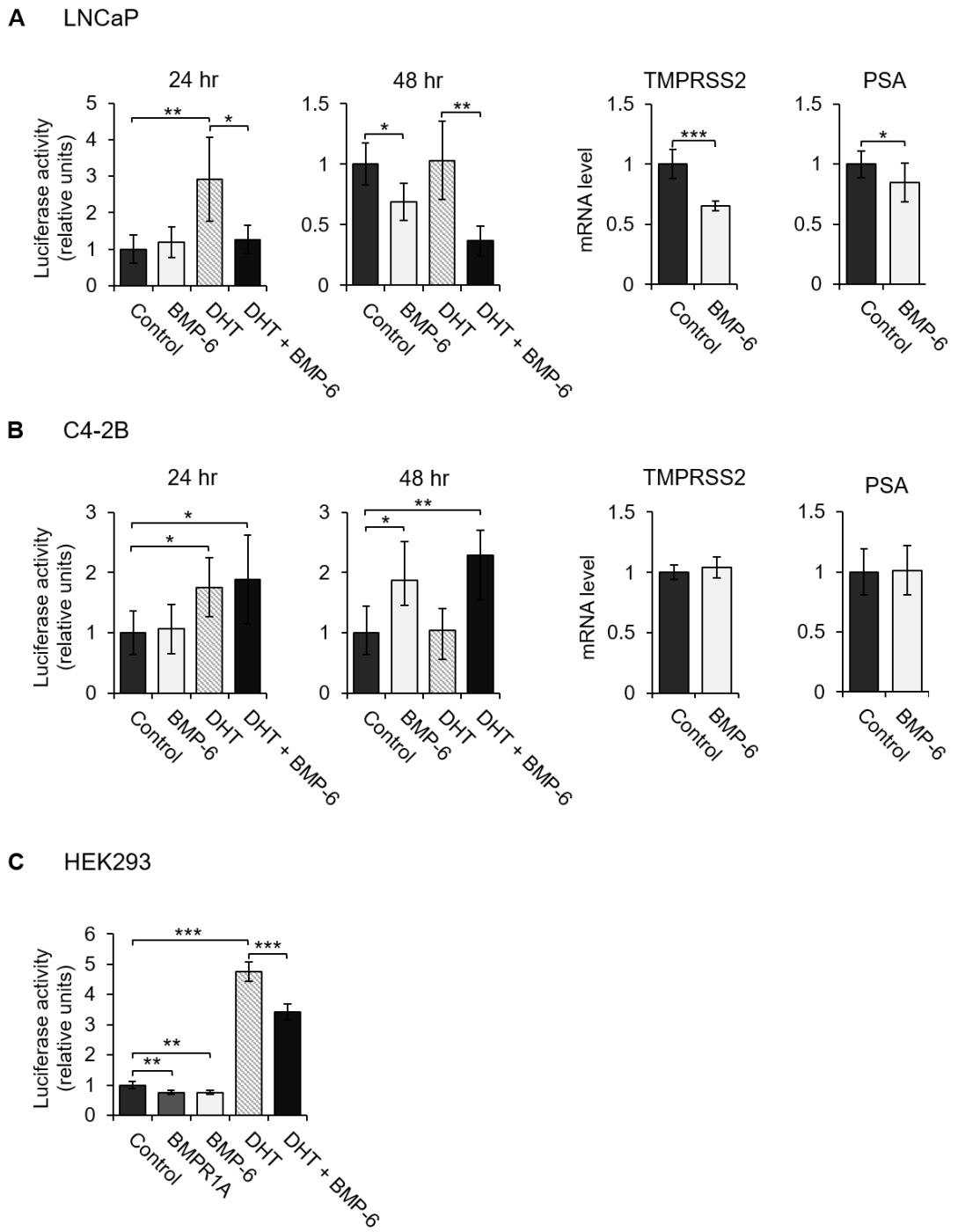


Figure 3.9. BMP-6 represses the transactivation of AR in LNCaP and HEK293 cells, but not in C4-2B cells. (A, B and C, **left** panels) For dual luciferase activity assays, cells were co-transfected with ARE-Luc and Renilla-Luc and treated with vehicle (methanol/BSA), BMP-6 (200 ng/ml), DHT (10 nM) or DHT and BMP-6 together for 24 or 48 hours. (A and B, **right** panels) Cells were treated with BMP-6 (200 ng/ml) for 24 hours and levels of indicated mRNAs were measured by qRT-PCR. Results are mean ±S.D of three experiments. *P < 0.05, **P < 0.01 or ***P < 0.001 vs. control.

cells upon BMP-6 treatment. However, the expression of each gene was moderately, but significantly, downregulated in LNCaP cells following BMP-6 stimulation, in contrast to the luciferase assay. One possible reason for the discrepancy in findings could be the technical difficulties I encountered whilst performing this assay. In particular, LNCaP cells proved difficult to transfect, meaning that the luciferase signal was often low in the unstimulated cells and subtle changes in signal were hard to detect. To combat this, in collaboration with Dr Carlo Rinaldi (Wood lab, University of Oxford), a HEK293-based androgen screening assay was developed to evaluate whether BMP-6 exhibited androgenic or anti-androgenic activity (Figure 3.9C). In brief, HEK293 cells were transfected with separate vectors encoding AR and the androgen-responsive luciferase reporter, then treated with or without BMP-6 and/or DHT for 24 hours. Again, the functionality of the system was confirmed with DHT treatment, suggesting that, in this setting, the receptor behaved similarly to AR in androgen-sensitive PCa cells. Interestingly, using this setup, treatment with BMP-6 significantly inhibited AR activity, both with and without androgens. Thus, reducing AR-mediated transcription may be the typical action of BMP-6 in androgen-sensitive cells, supporting the qPCR data shown earlier for LNCaP cells. Indeed, after 48 hours, the inhibitory effect of BMP-6 on AR activity was evident in LNCaP cells even without androgens present, further supporting this notion. Interestingly, at this same time point in C4-2B cells, BMP-6 treatment produced the opposite effect, stimulating AR activity in both androgen conditions. Hence, the effect of BMP-6 on the transcriptional activity of AR seemed to correlate with the androgen sensitivity of the cell lines.

Therefore, overall, it appeared that BMP-6 could modulate both the transcriptional activity of the AR and PCa cell growth. However, whether reduced AR signalling was driving the inhibitory effects on PCa cell growth remained unknown. To investigate this, the effect of BMP-6 on the expression of several AR-regulated cell cycle-related genes was examined.

3.2.5.2 Cell cycle control

It is well known that AR acts as a transcriptional activator to drive PCa cell growth. However, in the normal prostate, AR typically acts to inhibit cell growth by repressing the transcription of numerous cell cycle-related genes (Gao et al., 2016). In PCa cells, this anti-proliferative action of AR can still be stimulated by certain factors, for example, high concentrations of androgens. Therefore, the following work was completed to test whether BMP-6 represented another factor that could promote the transcriptional repressor activity of the AR and inhibit LNCaP cell proliferation.

Firstly, the inhibitory effects of DHT on the growth of LNCaP cells and the expression of several DNA replication genes were confirmed in my own experimental settings (data not shown). Subsequently, the expression of the same panel of DNA replication genes (i.e. LMNB1, MCM7 and FANCI) were examined in LNCaP and C4-2B cells following BMP-6 treatment (200 ng/ml) in androgen-deprived conditions for 24 hours. The expression of all three genes were significantly reduced in LNCaP cells upon addition of BMP-6, yet unaffected in C4-2B cells (Figure 3.10A), correlating with the observed effects of BMP-6 on AR activity and cell growth in both cell lines.

Additional regulators of the cell cycle that have been linked to both the BMP and AR signalling pathways are cyclin-dependent kinases (CDKs), their substrates, and CDK inhibitors. Briefly, CDKs phosphorylate downstream effector molecules to promote cell cycle progression, whereas their inhibitors negatively regulate this event by impeding the actions of CDKs, usually in response to some environmental stressor. One CDK inhibitor I decided to examine was p21, as it is a key regulator of cell growth and a common target of all TGF-β superfamily pathways (Pardali et al., 2005). Consistent with these published findings, both p21 mRNA and protein were upregulated following treatment with BMP-6 for 24 hours in LNCaP cells (Figure 3.10A, middle panel). However, neither p21 mRNA nor protein was affected by the same treatment in C4-2B cells (Figure 3.10B).

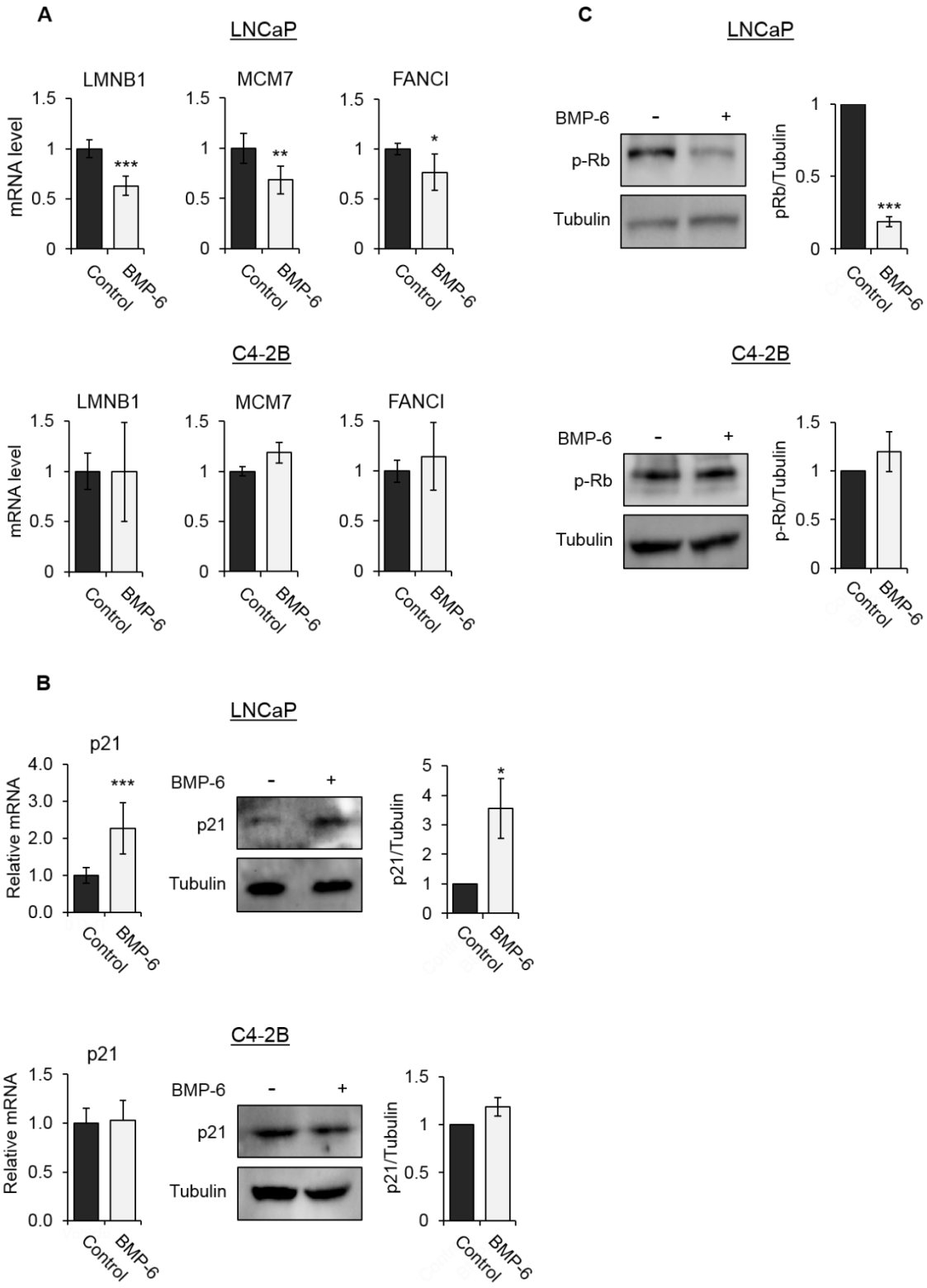


Figure 3.10. BMP-6 promotes a molecular profile reflective of cell cycle inhibition in LNCaP, but not C4-2B, cells. Cells were treated with BMP-6 (200 ng/ml) for 24 hours and the effects on the expression DNA replication genes **(A)**, p21 mRNA and protein levels **(B)** and Rb phosphorylation **(C)** was examined using qRT-PCR and western blotting. Results are mean ±S.D of three experiments. **P* < 0.05, ***P* < 0.01 or ****P* < 0.001 vs. control.

A common substrate of several CDKs is the tumour suppressor protein, Rb. Following phosphorylation, Rb promotes the transition of the cell cycle from G1 to S phase and is therefore an important regulator of cell growth. Since expression of the CDK inhibitor, p21, was altered by BMP-6, I decided to next examine the effects on Rb phosphorylation. Concordant with the results observed for p21, phosphorylation of Rb was reduced in LNCaP cells and unchanged in C4-2B cells following treatment with BMP-6 for 24 hours (Figure 3.10C). Thus, I wondered whether this difference in Rb phosphorylation could explain why LNCaP and C4-2B cells were responding differently to BMP-6 in terms of cell growth. To explore this further, I decided to examine the consequences of Rb loss on LNCaP cell growth for reasons discussed below.

3.2.5.3 Regulation of Rb

Mechanistically, when phosphorylated, Rb is activated and dissociates from the transcription factor, E2F, enabling E2F activation and cell cycle progression. Conversely, when de-phosphorylated, both proteins complex together to maintain cell cycle arrest. Therefore, to test the hypothesis that hypo-phosphorylated Rb is necessary to mediate the growth-inhibitory effect of BMP-6, Rb was knocked down in LNCaP cells. The rationale being that loss of Rb should mimic the effects of hyper-phosphorylated Rb i.e. increase E2F activity.

Cells were transfected with two different shRNAs targeting Rb (shRB#1 and shRB#2), then stable cell lines were generated through puromycin selection. Rb mRNA was reduced by more than 65% and Rb protein was almost undetectable when assessed by western blot in these cells compared to those transfected with non-targeting shRNA (Figure 3.11A). To confirm that cells deficient for Rb behaved similarly to those in published reports, the growth characteristics of these cells were then examined and compared to control cells. When cultured in the presence of androgens, all three cell lines displayed similar growth profiles (Figure 3.11B, left panel). However, in androgen-deprived conditions, cells deficient for Rb

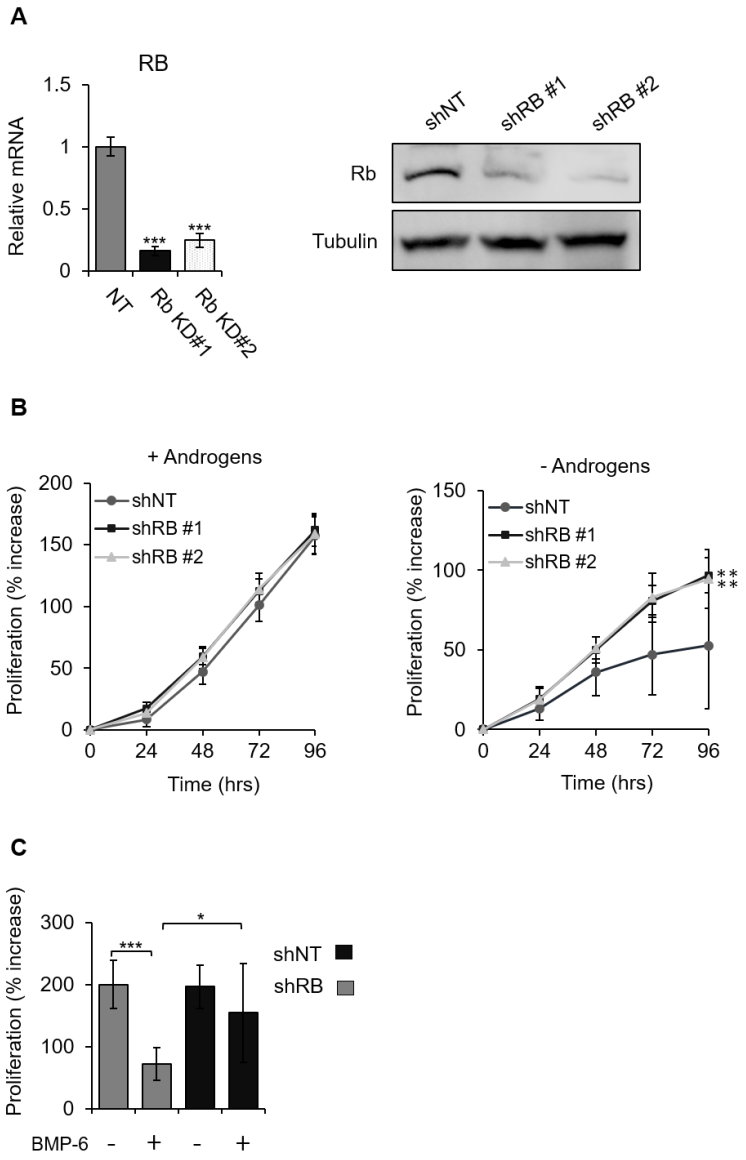


Figure 3.11. RB loss diminishes the growth-inhibitory effect of BMP-6 in LNCaP cells. **(A)** Levels of Rb mRNA and protein were examined in LNCaP cells transfected with shNT (control) or shRB (#1 and #2). **(B)** Cells from (A) were treated with DHT (0.1 nM; left panel) or vehicle (methanol; right panel) for 96 hours and cell proliferation examined over time. **(C)** Cells transfected with shNT and shRB#1 were treated with vehicle (BSA) or BMP-6 (200 ng/ml) and cell proliferation was assessed at 96 hours. (B and C) Red fluorescent images of cells were collected using the IncuCyte ZOOM™ and the number of red objects were quantified using the IncuCyte™ object counting algorithm. Cell proliferation is represented as the fold change in red objects (1/mm^2) between 0 and 96 hrs. Results are mean ±S.D of two (C) and three (B) independent experiments (8 replicates/per condition). **P* < 0.05, ***P* < 0.01 or ****P* < 0.001 vs. control at 96 hours.

had a significant growth advantage over their Rb-proficient counterparts (Figure 3.11B, right panel). Such findings have been previously reported and can be explained in terms of

altered Rb/E2F interactions in the two conditions. Briefly, as outlined above, in the presence of mitogenic signals such as androgens, Rb is phosphorylated and dissociates from E2F, whereas in their absence, the situation is reversed. Hence, only in the absence of androgens will loss of Rb provide a proliferative advantage. These results are consistent with the published literature (Sharma et al, 2012) and suggested to me that the Rb knockdown was successful in these cells.

Finally, after validating the Rb-deficient LNCaP cell lines, the effect of BMP-6 on the growth of these cells was examined. Despite both shRB cell lines behaving almost identically, those transfected with the first shRNA (shRB#1) were taken forward for further analysis as they exhibited slightly greater knockdown of Rb mRNA. These cells, along with control cells, were then treated with BMP-6 (200 ng/ml) for 96 hours and the effects on cell proliferation examined. As reported earlier, treatment with BMP-6 reduced LNCaP cell growth by more than 60% (Figure 3.11C). Interestingly, however, when Rb was knocked down, the inhibitory effect of BMP-6 was markedly reduced. Indeed, LNCaP cell proliferation was decreased by less than 25% in this scenario. Taken together, these results suggested that the growth-inhibitory effects of BMP-6 in LNCaP cells are mediated, at least in part, by Rb.

3.3. Discussion

As discussed earlier, a number of studies have investigated the role of BMP-6 in PCa progression; however, few conclusions have yet been drawn. Still puzzling is the frequent observation that BMP-6 expression increases as the disease advances, yet BMP-6 is most commonly reported to function as a tumour-suppressor, inhibiting PCa cell growth. In this chapter, I set out to reconcile these seemingly contradictory findings; to uncover the true effect of BMP-6 on PCa cell growth, the mechanisms underlying this response and how these findings relate to clinical data. Briefly, I have found that BMP-6 inhibits the growth of cells that are representative of a less aggressive form of PCa; that is, androgen-sensitive and slow growing. In light of these findings, together with the data I discuss in detail below, I propose the following model: within the bone microenvironment, elevated BMP-6 inhibits PCa cell growth, and, in turn, indirectly selects for more aggressive tumour cells with genetic aberrations conveying resistance to this inhibition (Figure 3.12).

3.3.1 BMP-6 can affect the growth of certain PCa cell lines

In this chapter, I found that BMP-6 could inhibit the growth of androgen-sensitive, but not androgen-insensitive, PCa cells. Specifically, I showed that C4-2B cell growth was largely unaffected by BMP-6 treatment, whereas LNCaP cell proliferation was inhibited by BMP-6, consistent with the published literature (Haudenschild et al., 2004; Kim et al., 2004; Dai et al., 2005; Lee et al., 2014). However, I also showed that DU145 cell growth and PC-3 cell proliferation were unchanged following BMP-6 treatment. These findings are slightly more contentious given that previous studies have demonstrated that BMP-6 can elicit a growth-inhibitory response in DU145 cells (Kim et al., 2004; Haudenschild et al., 2004) and in PC-3 cells (Kim et al., 2004). Although the reasons for these discrepancies are unclear, it is apparent that the culture conditions used in each of these studies differs to those employed here, hinting that the effects may be condition dependent.

Indeed, a condition dependent role for BMPs in regulating apoptosis has been previously described in breast cancer cells (Zabkiewicz et al., 2017). Furthermore, my own findings revealed that low concentrations of BMP-6 could promote LNCaP cell growth under conditions of cellular stress. Therefore, the ultimate effect of BMP-6 in PCa may be determined by the conditions within the tumour microenvironment. Whilst serum concentrations of BMP-6 have been reported, as have the levels of circulating testosterone, local concentrations of both likely differ in the bone marrow, rendering recapitulations using cell culture systems difficult. Moreover, numerous other secreted factors are also present in the bone environment which may affect BMP-6 signalling. Hence, although differences in culture conditions may help to explain the inconsistencies observed between my findings and the published literature, it is still difficult to conclude the effect of BMP-6 on PCa cell growth *in vivo*.

3.3.2 The bone environment mimics the effects of BMP-6 on PCa cell growth

To try and combat some of the issues outlined above, as well as develop an assay more reflective of physiological conditions, I also utilised a bone co-culture system. Overall, the effects of the bone stroma on PCa cell growth were largely consistent with the effects observed using exogenous BMP-6. That is, PC3 and C4-2B cell growth were unchanged when co-cultured with the stromal cells, whereas LNCaP cell growth was significantly reduced. However, increased p-Smad was only observed in LNCaP cells following the co-culture, suggesting that the bone stroma may stimulate BMP signalling in these cells alone. Nonetheless, it is still unclear whether BMP signalling, let alone BMP-6, is responsible for the inhibitory response observed in LNCaP cell growth using this setup. Therefore, further experiments, such as BMP-6 neutralisation or knockdown, are ultimately required. However, overall, these findings suggest that elevated BMP signalling in the bone environment is associated with decreased PCa cell growth.

Furthermore, these findings are largely consistent with the published literature. One study, utilising the same cell lines, similarly showed that co-incubation with bone stromal cells inhibited the growth of LNCaP but not PC-3 cells (Zhang et al*.,* 2011*).* However, the same study also reported that C4-2B cell growth was reduced in this setting. Whilst differences in culture conditions may again explain such discrepancies, it seems that *in vivo* studies are ultimately necessary to establish the effect of the bone environment on the growth of different PCa cell lines. Encouragingly, studies which have characterised the behaviour of PCa cell lines in murine bone have reported that whilst PC-3 and C4-2B cells both grow well, LNCaP cells grow very poorly following intraskeletal injection (Fisher et al., 2002; Jin et al., 2013). Although these studies did not examine the role of BMP-6 in regulating the growth response, they do support my findings, as well as my hypothesis, that signals within the bone environment normally act to inhibit PCa cell growth. Potential explanations as to why PC-3 and C4-2B cells may not respond in this manner are discussed in the sections below.

3.3.3 AR may be involved in regulating the growth-suppressive effects of BMP-6

Given that BMPs inhibit the growth of androgen-sensitive LNCaP cells but have no effect on the growth of the androgen-insensitive LNCaP subline, C4-2B, it is possible that the growth-suppressive effects of BMP-6 may be AR-dependent. Indeed, such a concept has been previously proposed for other BMPs. For instance, it has been shown that elevated BMP/Smad1 signalling inhibits LNCaP cell proliferation, whereas AR knockdown abrogates this effect (Qui et al., 2007). Likewise, the growth-inhibitory effect of BMP-7 in LNCaP cells has also been shown to depend on AR (Morrissey et al., 2010). Similar knockdown and overexpression studies could be performed to assess whether the effects of BMP-6 on PCa cell growth are also AR-dependent.

Further evidence that the AR plays a role in mediating the growth-suppressive effects of BMP-6, is the altered growth response observed in LNCaP cells to BMP-6 treatment

following AR inhibition. To recap, blocking AR signalling with bicalutamide reduced the inhibitory effect of BMP-6 on LNCaP cell growth, suggesting that AR activity is important in mediating the effects of BMP-6. One possibility could be that the AR regulates the expression of genes that encode components of the BMP-6 signalling pathway. Indeed, a previous study that examined the growth-suppressive effects of BMP-2 in LNCaP cells showed that androgens stimulated the expression of a specific BMP receptor at the cell surface; a response, the authors reasoned, which may have amplified the BMP-2 signal and thereby the growth-inhibitory effects of the ligand (Ide et al., 1997). Although further studies are required to determine the significance of these findings for this work, it is possible that crosstalk between the BMP-6 and AR signalling pathways is responsible for the inhibitory effects on PCa cell growth, and that alterations in AR signalling may therefore explain why certain cell lines display resistance to the effects of BMP-6.

3.3.4 BMP-6-induced growth inhibition is partially mediated by Rb

My data also revealed that Rb knockdown diminishes the growth-inhibitory effect of BMP-6 on LNCaP cell growth, suggesting Rb is necessary to elicit this response. Correspondingly, DU-145 cells do not express Rb and were not affected by BMP-6 treatment. However, the decrease in the p-Smad signal observed in these cells following stimulation with BMP-6 indicates that other components of the BMP pathway, in addition to Rb, may render these cells refractory to BMP-6-induced growth inhibition. One possibility is that negative regulators of BMP-6 are upregulated in DU-145 cells and inhibit downstream signalling. Indeed, the BMP inhibitor Noggin has been shown to be upregulated in these cells in response to BMP-6 (Haudenschild et al., 2004). Nonetheless, the lack of response exhibited by the Rb-proficient C4-2B and PC-3 cells, further signifies that Rb expression alone is not sufficient for BMP-6-mediated growth inhibition. Hence, in these cells, like the DU-145 cells, it is likely that additional regulators of the BMP-6 pathway may be altered.

As previously discussed, one well-characterised genetic alteration in PC-3 cells is their lack of AR expression. Considering the work presented in this chapter demonstrates that crosstalk exists between the BMP-6 and AR signalling pathways, it is possible that AR is required to mediate the effects of BMP-6 on growth. Indeed, other groups have reported that expressing AR in PC-3 cells renders them susceptible to the growth-suppressive effects of enhanced BMP/Smad1 signalling (Qui et al., 2007). A similar experiment could be performed with BMP-6 to explore whether any parallels exist with my work. Considering AR signalling is also altered in C4-2B cells, the above rationale could explain why BMP-6 does not inhibit growth in this cell line either. Another possibility concerns alterations in the regulation of Rb activity.

In LNCaP cells, decreased Rb phosphorylation correlates with BMP-6-induced growth inhibition; a response partially suppressed by Rb knockdown, suggesting that hypophosphorylated Rb may be important in mediating this event. Consistently, Rb phosphorylation is unaltered in C4-2B cells following BMP-6 treatment and correlates with no change in cell growth. This result further suggests that the machinery responsible for regulating this response may be altered in these cells. Indeed, expression of p21, an upstream negative regulator of Rb phosphorylation, was unaffected by BMP-6 treatment in C4-2B cells yet upregulated in LNCaP cells, consistent with literature reports (Haudenschild et al., 2004). Considering the BMP-6 effector proteins Smad1/5 are known promoters of p21 transcription (Pardali et al., 2005), it is possible that this regulatory pathway becomes aberrant in advanced PCa. Nonetheless, studies examining p21 expression in PCa have been non-conclusive, perhaps reflecting the heterogeneity of the disease. Instead, a more informative analysis could involve assessing whether a positive correlation exists between p-Smad and p21 in early versus late-stage PCa.

In terms of the role Rb may play in mediating the growth-suppressive effects of BMP-6, it is likely to involve regulation of the cell cycle. Indeed, Rb is key player in cell cycle control

and tumour growth. As outlined earlier, the primary function of Rb is to prevent unscheduled cell cycle entry by regulating a major G1 checkpoint, the transition to S-phase. To suppress cell growth, Rb inhibits the transcription of genes necessary for entry into S-phase. Mechanistically, this process involves active (i.e. hypophosphorylated) Rb associating with the E2F transcription factors to inhibit E2F activity and transcription indirectly, or the Rb-E2F repressor complex functioning together to actively repress transcription. Interestingly, it was recently suggested that AR may be involved in regulating the latter event. In a series of *in vitro* studies examining the anti-proliferative function of AR, the receptor was shown to inhibit PCa cell growth by recruiting hypophosphorylated Rb to several E2F-regulated DNA replication genes, repressing their transcription (Gao et al., 2016). One attractive hypothesis given these findings, along with those presented in this chapter, is that BMP-6 promotes the formation of this transcriptional repressor complex to inhibit LNCaP cell growth. Indeed, treatment with BMP-6 decreased Rb phosphorylation in this cell line, supporting this notion. This same treatment also increased AR protein levels; a difficult outcome to reconcile with the growth-inhibitory effects of BMP-6, but one which may make sense in this context, considering the authors noted that high levels of AR can enhance the repressor activity of the complex. Also supporting this theory is the observation that BMP-6 treatment downregulates the expression of several DNA replication genes also identified by this group as being regulated by the AR-Rb-E2F complex. Furthermore, no difference in expression of these genes was observed in C4-2B cells following BMP-6 treatment, correlating with the lack of growth effect also observed in these cells.

Future work could focus on examining the interaction dynamics of the components that make up the repressor complex in response to BMP-6, and the transcriptional consequences of this in PCa cells. For example, transcription of the AR-regulated DNA synthesis genes could be examined following both Rb knockdown and BMP-6 treatment to determine whether Rb contributes to the repression of these genes. Furthermore, chromatin

immunoprecipitation assays may also be useful in determining whether BMP-6 affects the recruitment of Rb to AR binding sites in the DNA synthesis genes, and whether AR and Rb complex together to repress transcription. The consequences of the above manipulations on cell cycle progression and cell proliferation could further be examined to highlight the role of this complex in mediating the functional effects of BMP-6 in PCa cells.

3.3.5 Clinical implications

Overall, the results presented in this chapter suggest that BMP-6 can inhibit the growth of PCa cells via a mechanism involving Rb, and potentially AR. However, these findings present a seemingly contradictory scenario; expression of BMP-6 reportedly increases as PCa progresses, yet I propose that this ligand primarily functions to supress tumour growth in late-stage PCa. An emerging role for BMPs in a process known as metastatic dormancy could provide an explanation.

As an event, metastasis culminates in the colonisation of distal sites by disseminated tumour cells (DTCs). Once here, cells undergo proliferative quiescence if faced with a non-permissive niche. In particular, PCa tumours commonly undergo prolonged periods of dormancy (Morgan et al., 2009). Various factors, present in this environment, have been highlighted as regulators of metastatic dormancy, including BMPs. For example, BMP-4 can promote dormancy of mouse mammary DTCs in the lung, whereas BMP-7 induces quiescence of prostate tumour cells in bone (Sosa et al., 2014). BMP-6 may also drive this process in bone-metastasised PCa cells, consistent with my findings that BMP-6 inhibits PCa cell growth, as well as clinical observations that expression of this ligand is associated with advanced PCa. Indeed, cells that eventually overcome such inhibitory signals will evidently drive a more aggressive disease.

Reviewing the model I proposed earlier; that is, that PCa tumour cells with genetic aberrations conveying resistance to BMP-6-induced growth inhibition will be selected for in

the bone microenvironment, it is now possible to speculate as to what these alterations may be.

Although unproven, I have presented evidence to suggest that AR signalling may be involved in mediating the growth-suppressive effects of BMP-6. If true, one would expect AR signalling to become altered as PCa progresses, which we know to be the case. However, as most men with PCa who develop skeletal metastases receive ADT, it is difficult to disentangle the influence of anti-androgens from that of the bone environment. Although, interestingly, the bone environment was recently suggested to accelerate the development of hormone-refractory PCa, as men with skeletal metastases reportedly develop CRPC faster than those without (Lee et al., 2014). Thus, perhaps elevated BMP-6 in this environment promotes such a phenotype. Additional alterations we may expect, given my findings and the proposed model, include those in the Rb pathway.

In terms of Rb expression, unlike in countless other cancers where loss of this key tumour suppressor protein is associated with cancer development, in PCa, Rb loss correlates with disease progression. For instance, loss of Rb is rarely observed in localised PCa yet overrepresented in metastatic disease, and moreover, is associated with reduced recurrence-free survival after prostatectomy. It has recently been suggested that perturbations in the Rb pathway may be driven by anti-androgen therapy. Indeed, as discussed earlier, it is only in the absence of androgens that loss of Rb provides a proliferative advantage to PCa cells, perhaps explaining why Rb loss is also associated with the development of CRPC. However, another explanation proposed here is that the bone environment applies additional selective pressure on metastatic PCa tumours, providing Rb-deficient cells with a growth advantage over their Rb-proficient counterparts - an advantage which may be further enhanced by ADT. Further work to explore this theory could involve examining the growth of Rb-deficient PCa cells *in vivo*. Indeed, LNCaP cells exhibit notoriously poor take rates when injected intratibially, perhaps due to growth-

inhibitory signals (e.g. BMP-6) within the bone microenvironment. Competition assays between Rb-proficient and Rb-deficient PCa cells could also be performed to assess whether the bone stroma selects for the latter, whereas knockdown experiments could help to determine the involvement of BMP-6.

Mechanistically, the theory proposed here raises two possibilities regarding Rb loss in PCa. Either, loss of Rb occurs prior to metastasis, or, alterations in Rb expression occur in situ. In support of the former theory, it has recently been shown using murine models of PCa that Rb loss produces a pro-metastatic phenotype in tumour cells, characterised by an increase in their migratory and invasive capacity (Thangavel et al., 2017). Hence, these findings suggest that the overrepresentation of Rb loss observed in metastatic PCa may be a result of Rb-deficient cells possessing increased metastatic potential (i.e. loss of Rb occurs prior to, and drives, metastasis). I propose that the bone environment also contributes to Rb loss by selecting for, and amplifying, these newly-metastasised Rb-deficient cells. Nonetheless, the observation that it takes several years for patients to develop bone metastases following primary tumour resection, casts doubt upon this explanation. Indeed, it seems questionable that loss of Rb enables cells to overcome the growth-inhibitory signals (i.e. elevated BMP-6) within the bone microenvironment *and* occurs prior to metastasis given the lag phase separating the entry of Rb-deficient tumour cells into the bone stroma and their outgrowth. Alternatively, it is possible that additional growth-inhibitory signals exist within the bone environment, and more time may be required for cells to acquire the appropriate mutations, in addition to Rb loss, to overcome these barriers.

The above notion represents a well-studied concept in cancer evolution: mutations that confer a survival advantage are selected for and reflect specific selective pressures within the tumour microenvironment. Indeed, this event has previously been described in breast cancer. A gain-of function mutation enabling breast cancer cells to overcome inhibitory BMP

signals and exit dormancy is specifically associated with metastatic relapse in the lung; a niche containing high levels of bioactive BMP, but not the brain; a site devoid of such activity (Gao et al, 2012). Analogous experiments could be performed here to similarly analyse whether Rb loss promotes metastatic outgrowth of PCa tumours in sites of high BMP-6 versus low, and whether an inverse relationship between Rb and BMP-6 expression exists in patient tissue. Moreover, genetic profiling of PCa tumours could reveal additional mutations required to overcome anti-metastatic signals and undergo reactivation in the bone.

To my knowledge, my work is the first to suggest Rb loss as a mechanism to escape BMP-6-induced dormancy of PCa tumour cells in the bone. Such findings could reconcile firstly, why the most common effect reported for BMP-6 is growth-inhibition, yet expression of the ligand increases as PCa progresses, and secondly, why Rb loss also correlates with PCa progression, but is normally associated with cancer development. Not only do these findings highlight potential mechanisms underlying the development of metastatic dormancy and reactivation, but castration-resistance also, as Rb loss has been shown to drive this process *in vitro* (Sharma et al., 2010). Thus, the clinical implications of my findings are far-reaching. Moreover, although the work presented in this chapter indicates that BMP-6 is an unlikely therapeutic target for the treatment of advanced PCa, increased BMP signalling may prove useful in predicting those patients likely to develop Rb-deficient tumours. Such a tool could enable the identification of high-risk patients requiring aggressive and more targeted treatment strategies. Indeed, it has recently been shown that breast cancer tumours lacking Rb respond particularly well to chemotherapy (Witkiewicz and Knudsen, 2014). Hence, by understanding of the ways in which microenvironmental factors such as BMP-6 influence the evolution of metastatic PCa, we may be able to identify biomarkers predictive of patient outcome and develop more targeted therapies to prevent disease progression.

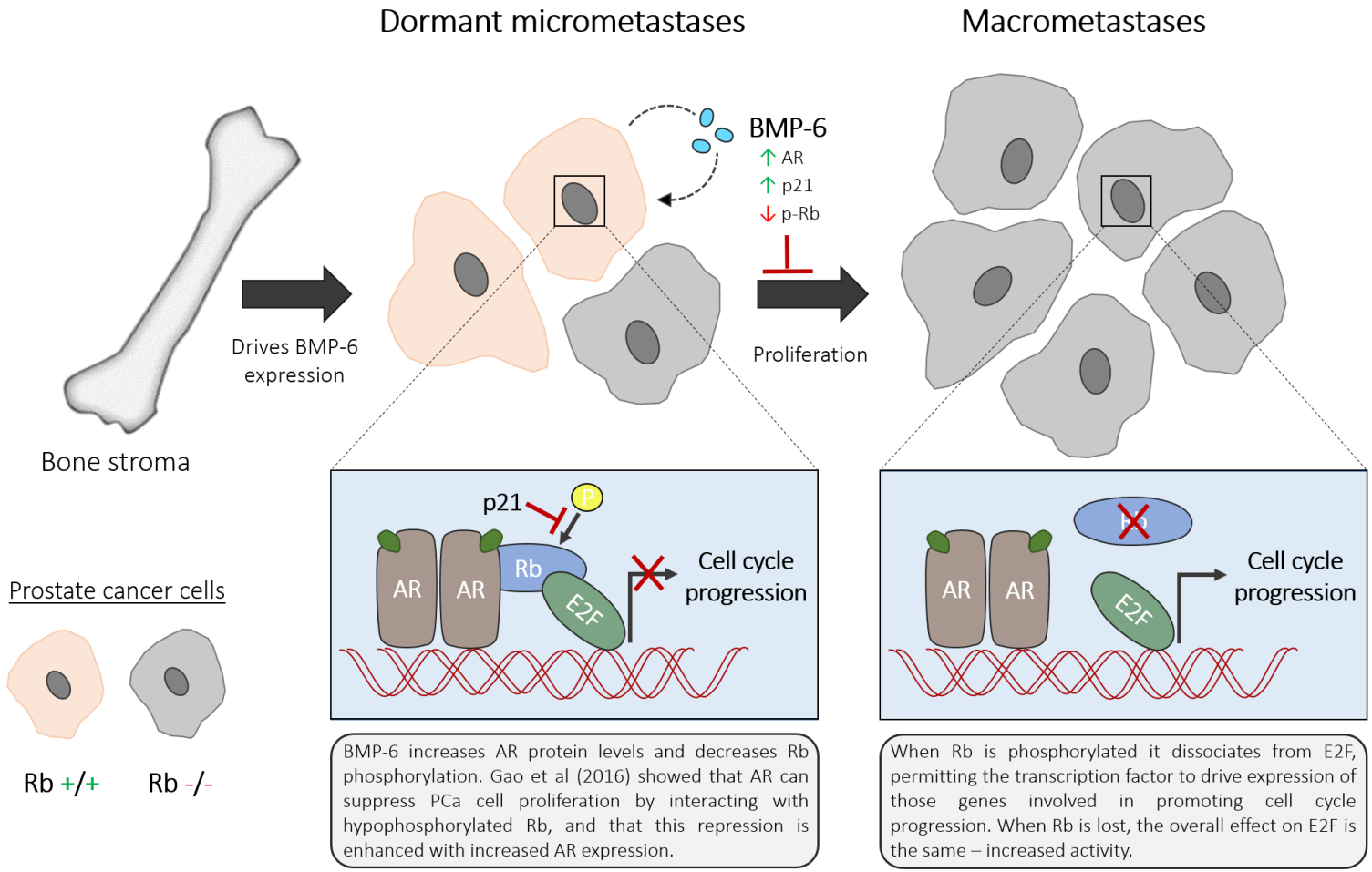


Figure 3.12. Speculative model to explain metastatic dormancy and reactivation of PCa tumour cells in the bone. The bone stroma has been shown to stimulate BMP-6 secretion in PCa cells (Lee et al., 2014). My findings suggest that BMP-6 inhibits PCa cell growth via a mechanism involving Rb and AR, which could involve the formation of the AR-Rb-E2F transcriptional complex as proposed by Gao et al (2016). Thus, loss of Rb, a frequent event in advanced PCa, may enable cells within the bone environment to escape this inhibition and drive metastatic outgrowth.

CHAPTER 4

RESULTS

4. Investigating the role of the PAT4-mTORC1 signalling axis in PCa

4.1. Introduction

4.1.1 Amino acid sensing in the cell

Following the discovery that the Rag GTPases play a key role in amino acid-dependent mTORC1 activation, recent research efforts have focussed on elucidating the mechanisms by which amino acid availability is sensed within the cell. Findings from several studies have suggested that independent mechanisms exist to detect amino acids in the LEL lumen as well as in the cytoplasm. For example, GATOR1, a component of the multiprotein complex GATOR which functions as a central node in the sensing of cytosolic leucine and arginine, inhibits mTORC1 activity by serving as a GAP for Rag A and Rag B, whereas GATOR2 promotes mTORC1 activity by inhibiting GATOR1 (Bar-Peled et al., 2013). Other cytosolic amino acid sensors stimulate mTORC1 signalling, in part, by modulating GATOR2 activity. For instance, Sestrin2 and CASTOR1 interact with and inhibit GATOR2 function under leucine and arginine deprivation, respectively (Chantranupong et al., 2014 and 2016; Parmigiani et al., 2014; Saxton et al., 2016; Wolfson et al., 2016). GATOR-independent mechanisms of amino acid signalling in the cytoplasm have also been identified. For example, Leucyl-tRNA synthetase (LRS), a GAP for Rag D, positively regulates mTORC1 activity in a leucine-dependent manner (Han et al., 2012). Similarly, folliculin and its interacting protein, FNIP, activate mTORC1 in the presence of amino acids by serving as a GAP for RagC and RagD (Petit et al., 2013; Tsun et al., 2013).

In terms of amino acid sensing in the LEL lumen, Rag GTPase activity is modulated via a mechanism dependent on the LEL-resident v-ATPase (Figure 1.7). Amino acids induce a conformational change in the v-ATPase that stimulates the guanine-nucleotide exchange factor (GEF) activity of the Ragulator towards Rag A and Rag B, promoting mTORC1

activity (Zoncu et al., 2011; Bar-Peled et al., 2012; Jewell et al, 2013). Given that the v-ATPase is located on the LEL surface where it associates with several other mTORC1 regulatory components, and that these interactions are sensitive to changes in amino acid levels, the v-ATPase has been suggested as the intra-LEL sensor of amino acids (Zoncu et al., 2011). However, an alternative theory proposes that certain intracellular transporters may also function to sense amino acid availability within this compartment.

4.1.2 Amino acid sensing and the PATs

The solute-linked carrier (SLC) proteins comprise a large family of membrane proteins that facilitate the transport of a wide array of molecules, including amino acids. Although several members of the SLC family have been shown to regulate mTORC1 activity (Nicklin et al, 2009), many do so simply via the transport of amino acids into the cell. However, a number of SLC transporters have been identified on LEL membranes, raising the possibility that certain members may be involved in amino acid sensing from this compartment. For example, SLC38A9, an amino acid transporter located at the LEL surface, associates with the Rag-Ragulator-v-ATPase complex and is required for arginine-dependent activation of mTORC1 (Jung et al., 2015; Rebsamen et al., 2015; Wang et al., 2015). Similarly, SLC36A1 (PAT1), another amino acid transporter primarily localised to LELs, interacts with the Rag GTPases and plays a key role in the recruitment of mTOR to LEL membranes (Ögmundsdóttir et al., 2012). Furthermore, PAT1 is required for amino-acid-dependent mTORC1 activation and drives cell growth in cancer cell lines and *Drosophila* (Heublein et al., 2010). Moreover, since some amino acid transporter family members, like *Drosophila* PATH and human PAT4, exhibit low amino acid transport capacity and still appear to regulate mTORC1, it has been suggested that other transporters in the SLC36 and SLC38 families may employ similar mechanisms to control mTORC1 activity. This has led to the proposal that these SLC proteins act as “transceptors”; transporters which function as amino acid receptors by inducing signal transduction rather than transport (Goberdhan et al., 2005; Goberdhan, 2010). It has even been

suggested that certain transceptors may mediate amino acid sensing from intracellular compartments other than LELs.

4.1.3 The multi-hub model of mTORC1 signalling

Until recently, the majority of studies have suggested that amino acids signal to mTORC1 via the Rag GTPases, and moreover, that LELs are the sole platform from which amino acid availability is measured and mTORC1 is activated. However, the finding that Arf1 and Rab1A, regulators of membrane trafficking, modulate the activity of a Golgi-localised mTORC1 complex via Rag-independent mechanisms indicates that a multi-hub model of mTORC1 regulation may exist (Jewell et al., 2015; Thomas et al., 2014). Indeed, PAT4 is a high-affinity, low-capacity member of the SLC family of transporters, implicated in amino acid signalling to mTORC1 from this compartment. For example, in HCT116 colorectal cancer cells, PAT4 is predominantly localised to the trans-Golgi network where it interacts with Rab1A, Raptor, and mTOR (Fan et al., 2016; reviewed in Goberdhan et al., 2016). In these cells, PAT4 regulates an alternative form of mTORC1 that preferentially targets 4E-BP1 and is resistant to rapamycin treatment. Furthermore, glutamine and serine have been shown to stimulate rapamycin-resistant mTORC1 activity in a PAT4-dependent manner. Altogether, these findings have led to the proposal that distinct mTORC1 complexes, located in separate subcellular compartments with different target specificities, are present within cancer cells and may control different cellular functions. For instance, PAT4 has recently been shown to play a role in regulating EV signalling in colorectal cancer cells.

4.1.7 The PAT4-mTORC1 axis regulates exosome signalling in colorectal cancer cells

As discussed earlier, recent work in our lab has demonstrated that exosomes are not only formed within LEL structures in the cell. Another endocytic compartment, marked by the recycling endosomal marker Rab11a, has been shown to generate and secrete Rab11a-positive ILVs via a mechanism that is conserved between human cancer cell lines and *Drosophila*. Moreover, vesicles formed within these Rab11a-compartments are

preferentially secreted from tumour cells following either glutamine depletion, PAT4 knockdown or inhibition of mTORC1 activity. Furthermore, this so-called 'exosome switch' results in increased secretion of exosomes that can promote pro-tumorigenic functions in recipient cells and may, therefore, mediate adaptive responses of tumours to microenvironmental stress.

4.1.8 PAT4 expression and EV signalling are altered in PCa

In PCa, EVs are being increasingly implicated in processes that promote disease progression. For example, EVs secreted from PCa cells have been shown to promote cell proliferation (Soekmadji et al., 2016), angiogenesis (Naito et al., 2017), metastasis (Bijnsdorp et al., 2013; Liu et al., 2016), immune evasion (Lundholm et al., 2014*)*, and drug resistance (Corcoran et al., 2012); however, the regulatory mechanisms underpinning these changes in the secreting cells remain poorly characterised. In addition, mutations in the PI3K/AKT/mTOR pathway are highly prevalent in PCa (Taylor *et al.*, 2010). Indeed, PAT4 expression is upregulated in PCa (Dan Stevens; Figure 1.8), and PAT4 gene changes are reportedly more common in aggressive PCa and associated with biochemical and metastatic recurrence (Taylor *et al*., 2010). Hence, establishing the role PAT4 plays in regulating PCa cell behaviour could have important clinical implications.

4.1.8 Aims and objectives

i. Characterise the function of PAT4 in PCa cells
ii. Establish conditions and an isolation method to purify EVs from PCa cell lines
iii. Investigate the role of the PAT4-mTORC1 signalling axis in regulating EV secretion

4.2 Results

4.2.1 PAT4 regulates mTORC1 signalling in PCa cells

In order to characterise the function of PAT4 in PCa, I decided to focus on investigating the potential role this transporter plays in regulating mTORC1 signalling and cell growth, given the previous findings reported for PAT4 in other cancer cell lines (Heublein et al., 2010; Fan et al., 2016). Experimentally, the approach I took involved silencing PAT4 expression, then assessing changes in the phosphorylation status of certain mTORC1 substrates, as well as the growth of PCa cells in culture. The VCaP cell line was chosen for this analysis as it is an androgen-sensitive PCa cell line and, given the crosstalk that has been reported between mTORC1 and the AR signalling pathway, I also wanted to investigate the effects of androgen stimulation on PAT4 function. Initially, to determine the optimal conditions required to knockdown PAT4, VCaP cells were transduced with different volumes of lentiviral medium containing viral particles encoding shRNA targeting *PAT4*. Following transduction, I decided to culture the cells for five days before assessing PAT4 expression levels, reasoning that VCaP cells grow very slowly and may, therefore, require a longer incubation period in which to turnover PAT4.

To determine the efficacy of the knockdown, both RNA and protein were isolated from VCaP cells and the expression of PAT4 was examined. Transduction of cells with shPAT4 decreased the expression of *PAT4* mRNA by approximately 30-60%, compared to cells treated with the shNT control (Figure 4.1A); whereas PAT4 protein levels appeared even further reduced in all of the four conditions tested (Figure 4.1B, lower panel). Reasons for the discrepancy between the levels of *PAT4* mRNA and protein were unclear, although one possible explanation could be the mechanism of gene silencing. For example, depending on the complementarity of the shRNA and mRNA sequences, shRNA can inhibit gene

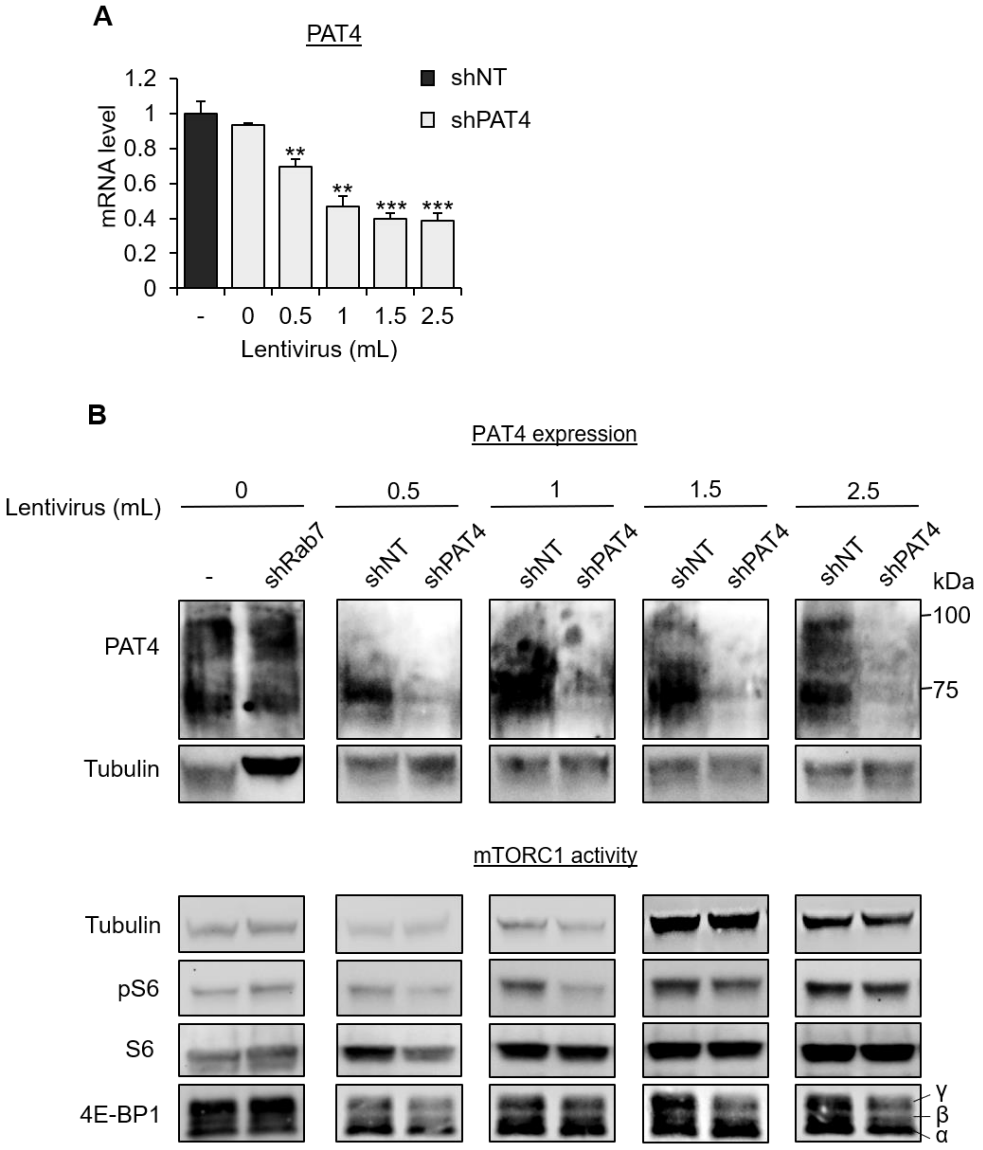


Figure 4.1. Establishing conditions to knockdown PAT4 in VCaP cells. **(A)** VCaP cells were transduced with the indicated volumes of shNT or shPAT4 for 5 days before the level of PAT4 mRNA was assessed via qRT-PCR. Results are mean ±S.D of three experiments. **P < 0.01 or ***P < 0.001 vs. control. **(B)** Levels of PAT4 protein and mTORC1 activity were assessed in the lysates derived from the cells in (A) via western blotting. shRab7 was used as a negative control.

expression by employing one of two strategies: promoting the cleavage of target mRNA or preventing the translation of target mRNA. In this case, it appeared that the latter mechanism may dominate considering shRNA treatment decreased the levels of PAT4 protein more than *PAT4* mRNA. Nonetheless, overall, the results indicated that even the lowest volume of lentivirus tested could successfully silence PAT4 protein expression.

Seeking additional confirmation, I then decided to assess mTORC1 activity in the same samples to determine (i) whether knocking down PAT4 altered mTORC1 signalling in VCaP cells and, if it did, (ii) the optimal volume of lentivirus to use in future experiments to investigate PAT4 function (i.e. that which produced the greatest effect).

To assess the effect of PAT4 knockdown on mTORC1 activity, the phosphorylation status of two well-characterised downstream mTORC1 targets, S6 and 4E-BP1, were examined. Overall, although PAT4 knockdown appeared to reduce the phosphorylation of S6 and 4E-BP1 in several of the conditions tested, the effects did not seem to correlate with the lentiviral potency (Figure 4B, lower panel). For instance, whilst hyperphosphorylated 4E-BP1 (the uppermost band γ-band) was reduced in all lanes following PAT4 knockdown, treatment with the shNT control also reduced this band. Hence, it was possible that the shNT construct was negatively affecting mTORC1 activity. However, when the shNT dose was increased, 4E-BP1 phosphorylation increased, suggesting that factors other than the construct may have been responsible for the variation in basal mTORC1 activity. One important point to note is that the culture medium was not replenished prior to protein isolation, hence decreased nutrient availability may explain the reduction in mTORC1 signalling observed here. In addition, technical difficulties were encountered when plating the VCaP cells, thus variations in cell number between the conditions could also potentially explain the disparity in basal mTORC1 activity. Nonetheless, the two conditions in which 4E-BP1 phosphorylation was minimally altered following treatment with shNT, yet inhibited following treatment with shPAT4, were those using 1.5 and 2.5 mL of lentivirus. Therefore, further experiments were performed using the lower of the two volumes to try and limit any potential off-target effects caused by the constructs.

4.2.2 PAT4 expression is regulated by androgens

As discussed in the introduction (section 1.2.1), androgens are the main regulators of cellgrowth within the normal prostate and play a key role in PCa development and disease

progression. One way in which androgens regulate PCa cell growth is by enhancing mTORC1 activity. Since I had shown that PAT4 knockdown inhibited mTORC1 activity in VCaP cells, I decided to examine whether androgens regulated PAT4 expression, in turn. To test this, the effects of androgens on *PAT4* mRNA were assessed in VCaP cells and in another androgen-sensitive PCa cell line, LNCaP, for comparative analysis. The concentration of androgens employed for this analysis (0.1 nM) was chosen as it represents the reported concentration at which peak androgen-mediated proliferation occurs in these cell lines. Hence, I reasoned that if PAT4 expression was regulated by androgens, then this concentration would provoke the greatest change in *PAT4* mRNA, allowing any differences to be easily detected. In VCaP cells, after 48 hours, androgen treatment significantly increased the expression of PAT4 mRNA by ~1.7 fold, similar to the increase observed in PSA (Figure 4.2A, left panel). Moreover, given that PSA is an androgen-regulated gene target, this result confirmed that the treatment conditions I had employed were sufficient to stimulate AR activity in these cells. Conversely, expression of PAT1 was unaffected by androgens. Considering both PAT4 and PAT1 are ubiquitously expressed members of the PAT family, these findings allude to a specific role for PAT4 in PCa. In addition, expression of SLC1A5, another glutamine-sensitive transporter upregulated in PCa, was increased following androgen treatment, consistent with the published literature and further supporting the PAT4 data. However, in LNCaP cells, PAT4 levels were unaffected by androgen treatment, despite PSA and SLC1A5 levels having increased (Figure 4.2A, right panel). Hence, it seemed that PAT4 expression was differentially regulated between the two cell lines.

Next, to assess whether the increase I had observed in *PAT4* at the mRNA level translated into increased expression at the protein level, western blot analysis was performed. However, before PAT4 expression could be examined in the experimental settings, conditions were first established to ensure PAT4 protein could be easily observed using this technique. As PAT4 is a transmembrane protein, difficulties detecting this protein via

western blotting have been reported using protein isolated from other cell lines (Shih-Jung Fan, personal communication). Indeed, purification of integral membrane proteins, such as PAT4, via gel electrophoresis is a common and significant problem. Primarily responsible is the hydrophobic nature of these proteins, rendering them prone to protein precipitation or to become trapped in the gels mesh. Measures to overcome these issues include; using appropriate detergents, reducing agents and temperatures to ensure complete sample denaturation and solubilisation, and to prevent protein aggregation. To this end, samples were prepared in a number of different ways by altering several of these parameters. For instance, high temperatures have been noted to promote membrane protein aggregation, therefore samples were heated at lower temperatures and incubated for longer periods of time to account for this (70ºC for 15 minutes or 37ºC for 30 minutes). In addition, samples were also treated with or without the addition of urea (8 M), as urea can reportedly facilitate solubilisation of the hydrophobic components of transmembrane proteins. Finally, the samples were prepared in either reducing or non-reducing conditions (+/- 10% β-mercaptoethanol (v/v)), to assess whether enhanced protein denaturation could influence the detection of PAT4.

The results of this analysis indicated that detection of PAT4 was influenced by all three of these parameters (Figure 4.2B). For example, in terms of temperature, PAT4 protein was only clearly detectable when the samples were heated at the lower of the two temperatures tested (37ºC for 30 minutes*).* Hence, it is possible that at higher temperatures PAT4 may form aggregates too large to enter the gels matrix, potentially explaining the absence of PAT4 signal in the lanes where samples were heated at 70ºC for 15 minutes. The addition of urea also seemed to enhance the PAT4 signal (compare lanes 1 vs 2 and 5 vs 6), as did the absence of β-mercaptoethanol (compare lanes 1 vs 5 and 2 vs 6). Hence, these findings indicated that protein samples prepared with non-reducing sample buffer, plus the addition of urea (8 M), and then heated at 37ºC for 30 minutes, would provide the optimal settings to examine PAT4 protein levels via western blotting. Implementing these conditions, the

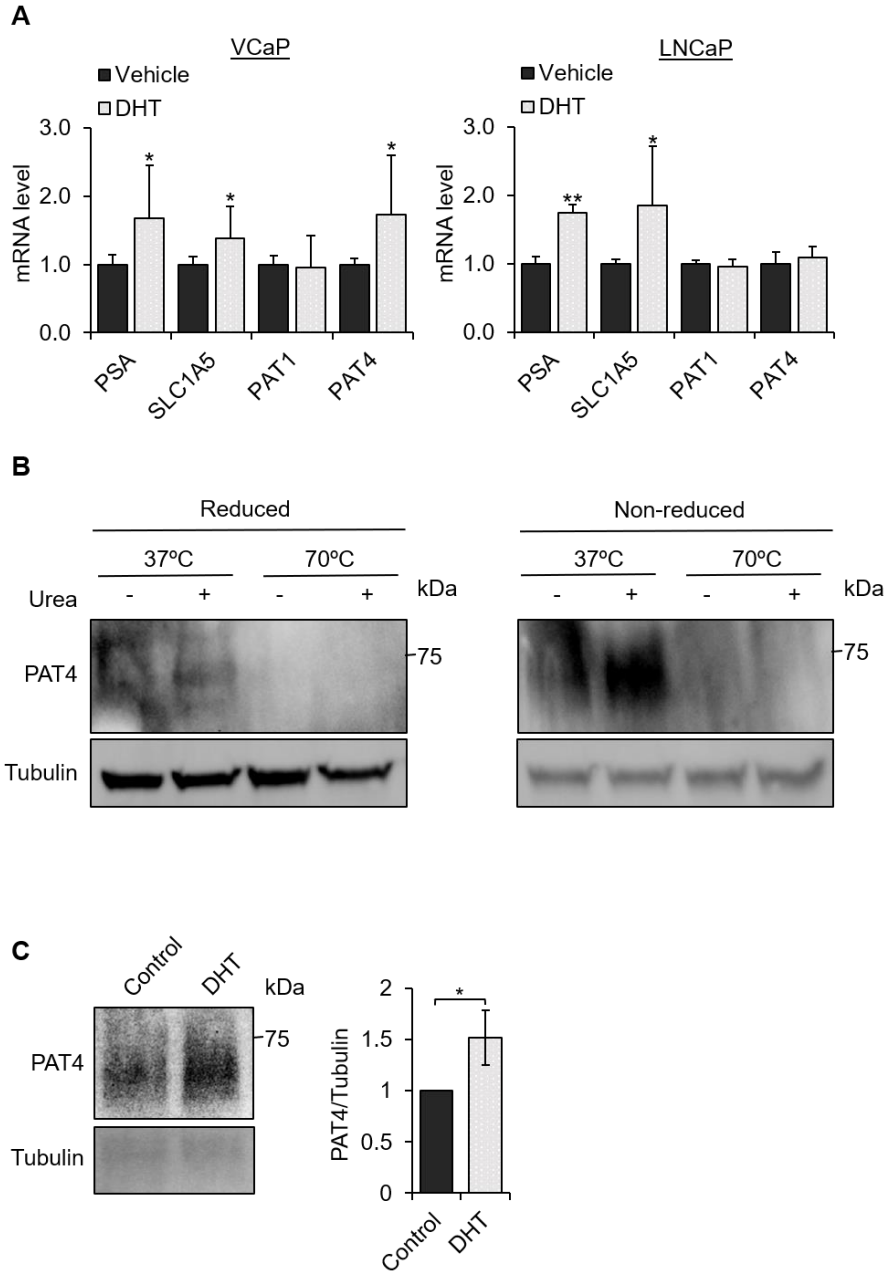


Figure 4.2. DHT stimulates PAT4 expression in VCaP cells. **(A)** VCaP and LNCaP cells were treated with vehicle (MeOH) or DHT (0.1 nM) for 48 hours before the levels of PSA, SLC1A5, PAT4 and PAT1 were examined via qRT-PCR. **(B)** VCaP cell lysate (50 µg) was subjected to several different solubilisation, reducing, and/or heating steps before PAT4 protein was assessed via western blotting. **(C)** VCaP cells were treated with vehicle (MeOH) or DHT (0.1 nM) for 48 hours before PAT4 protein was examined via western blotting. Results are mean ±S.D of three experiments. *P < 0.05 or **P < 0.01 vs. control.

effects of androgen treatment on the level of PAT4 protein was then assessed. Overall, androgen stimulation significantly increased PAT4 protein levels in VCaP cells after 48 hours, consistent with the results observed for *PAT4* mRNA (Figure 4.2C). Furthermore,

some of my preliminary data suggested that blocking AR signalling may reverse this effect (data not shown). Altogether, these findings indicate that PAT4 expression is positively regulated by DHT in VCaP cells, raising questions as to the role(s) PAT4 may play in mediating the functional effects of androgens in these cells.

4.2.3 Androgens promote PAT4-mediated PCa cell growth

Given that signalling via the AR and PAT4 have previously been shown to promote cancer cell growth, and that I have shown androgens drive PAT4 expression in VCaP cells, I decided to assess whether PAT4 was acting to mediate the stimulatory effects of androgens on PCa cell growth. To test this, VCaP cells were transduced with shNT or shPAT4 and cultured with or without androgens for a period of 48 hours, over which the effects on cell growth were assessed. This timeframe was chosen as I had previously shown following 48 hours of androgen treatment, PAT4 levels were significantly increased in VCaP cells. Therefore, I reasoned that any differences in cell growth following PAT4 knockdown may be easier to detect at this point. In control cells, the addition of DHT significantly increased cell growth at both 24 and 48 hours, consistent with the reported effects of androgens in these cells (Figure 4.3A). A similar trend was observed in PAT4-deficient cells, indicating that these cells respond appropriately to androgen stimulation and withdrawal, and moreover, that PAT4 expression alone is not sufficient to mediate the effects of androgens on PCa cell growth. However, knockdown of PAT4 significantly decreased androgen-mediated PCa cell growth, suggesting that PAT4 expression is necessary for maximal androgen-induced growth stimulation. Interestingly, knockdown of PAT4 had minimal effects on basal VCaP cell growth, suggesting a certain level of specificity for PAT4 in mediating androgen signalling in these cells. Nonetheless, after 48 hours there was a subtle but significant decrease in VCaP cell growth following PAT4 knockdown, indicating that, in the longer term, PAT4 may alter cell growth via androgen-independent mechanisms also.

To further understand the ways in which PAT4 mediates the effects of androgens on PCa cell growth, the effect of PAT4 knockdown on mTORC1 activity was examined in VCaP cells cultured with and without androgens for 48 hours (Figure 4.3B). In control cells, treatment with androgens stimulated mTORC1 activity, as evidenced by the increase in p-S6 and hyperphosphorylated 4E-BP1 (lane 1 vs 3, γ-band), consistent with findings reported in the published literature. Conversely, the same effect was not observed in PAT4-deficient cells (lane 2 vs 4) indicating that, as with cell growth, PAT4 is necessary to induce maximal androgen-induced mTORC1 activity. In addition, PAT4 knockdown also inhibited basal mTORC1 activity (lane 1 vs 2), suggesting PAT4 may regulate mTORC1 signalling via both androgen-dependent and -independent mechanisms. However, upon further inspection, it appeared that PAT4 loss did not inhibit total mTORC1 activity. Whilst the levels of hyperphosphorylated 4E-BP1 were reduced following the knockdown, p-S6 appeared unaffected, consistent with my previous observations (Figure 4B, lower panel), and those reported for PAT4 in colorectal cancer cells. Interestingly, when VCaP cells were treated with increasing concentrations of rapamycin, the opposite effect on mTORC1 signalling was observed. Specifically, the p-S6 signal was lost almost immediately, yet hyperphosphorylated 4E-BP1 persisted even when the cells were treated with very high concentrations of rapamycin (i.e. 100 nM), suggesting that hyperphosphorylated 4E-BP1 is regulated by rapamycin-resistant mTORC1 activity. Given that PAT4 knockdown selectively inhibited hyperphosphorylation of 4E-BP1, it is possible that PAT4 normally acts to positively regulate the rapamycin-resistant branch of mTORC1 in VCaP cells, similar to the regulation reported in colorectal cancer cells. Nonetheless, it remains unclear whether the effects of PAT4 knockdown on mTORC1 signalling underpin the growth inhibitory response observed in VCaP cells. Whilst VCaP cell growth is sensitive to mTORC1 inhibition (Figure 4D), further studies are ultimately warranted.

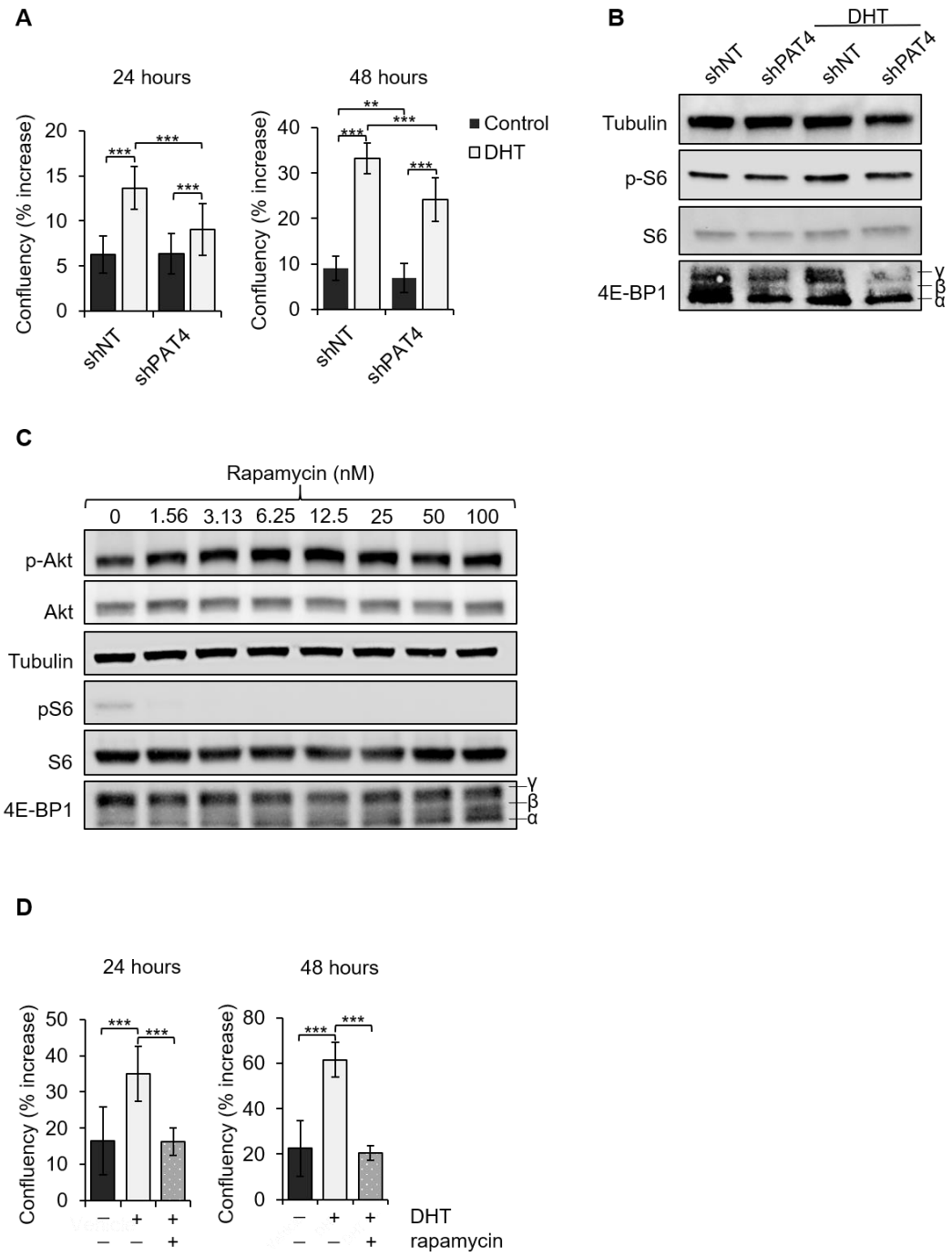


Figure 4.3. PAT4 knockdown inhibits VCaP cell growth and mTORC1 activity. **(A)** VCaP cells were transduced with shNT or shPAT4 for 5 days then treated with vehicle (meOH) or DHT (0.1 nM) for up to 48 hours. Phase-contrast images were collected every two hours using the IncuCyte ZOOM™ and cell confluency calculated using the IncuCyte™ software. Cell growth is represented as the fold change in percentage confluency between 0 and 24 or 48 hours. **(B)** VCaP cells were treated the same as in **(A)** and the effects on mTORC1 activity assessed via western blotting after 24 hours. **(C)** Western blot showing the dose-response of mTORC1 signalling in VCaP cells to rapamycin treatment after 24 hours **(D)** VCaP cells were treated with vehicle (DMSO/MeOH), rapamycin (100 nM) and/or DHT (0.1 nM) for up to 48 hours before the effects on cell growth were assessed as in **(A)**. Results are mean ±S.D of three experiments (n = 8 per condition). ***P < 0.001 vs. control.

4.2.4 Establishing media conditions to isolate EVs from PCa cells

As previously discussed, a novel role for the PAT4-mTORC1 signalling axis in regulating EV secretion has recently been demonstrated in colorectal cancer cells. Given that I have shown PAT4 also regulates mTORC1 activity in PCa cells, I next set out to address whether this signalling axis could similarly modulate the type of EVs released from PCa cells. I chose LNCaP and C4-2B PCa cell lines as the subjects for this analysis for several reasons. Firstly, both cell lines are closely related yet display different sensitivities to androgens, and since I have previously shown androgens to be important regulators of the PAT4-mTORC1 signalling in PCa cells, I reasoned that this important phenotypic difference could provide a useful comparison. Secondly, unlike the similarly androgen-sensitive VCaP cells, LNCaP cells grow considerably faster and therefore offer practical advantages in terms of conducting the large-scale exosome isolation experiments required for this analysis.

Initially, I began by trying to establish culture conditions that would allow me to isolate EVs from PCa cells grown in the absence of serum (i.e. EV depleted), whilst maintaining mTORC1 activity. Ensuring that mTORC1 activity was unaffected by the culture conditions during the period of EV collection was particularly important to realising the aim of this work; that is, to determine whether alterations in mTORC1 signalling alter the type of EVs secreted from PCa cells. Regarding the culture conditions, LNCaP and C4-2B cells were grown in either their recommended media or DMEM/F12, a media specially formulated for culturing cells in serum-free conditions (Arora, 2013). In addition, instead of FBS, which contains bovine-derived EVs, the media was supplemented with a cocktail of 1% insulin, transferrin and selenium (ITS) to maintain mTORC1 activity (Tauro et al., 2012). Cells were cultured in these conditions for 24 and 48 hours before the effects on 4E-BP1 and S6 phosphorylation and PCa cell growth were assessed (Figure 4.4).

Compared to cells grown in complete medium (i.e. plus 10% FBS), culturing cells in media supplemented with ITS had no observable effect on the phosphorylation of 4E-BP1 or S6

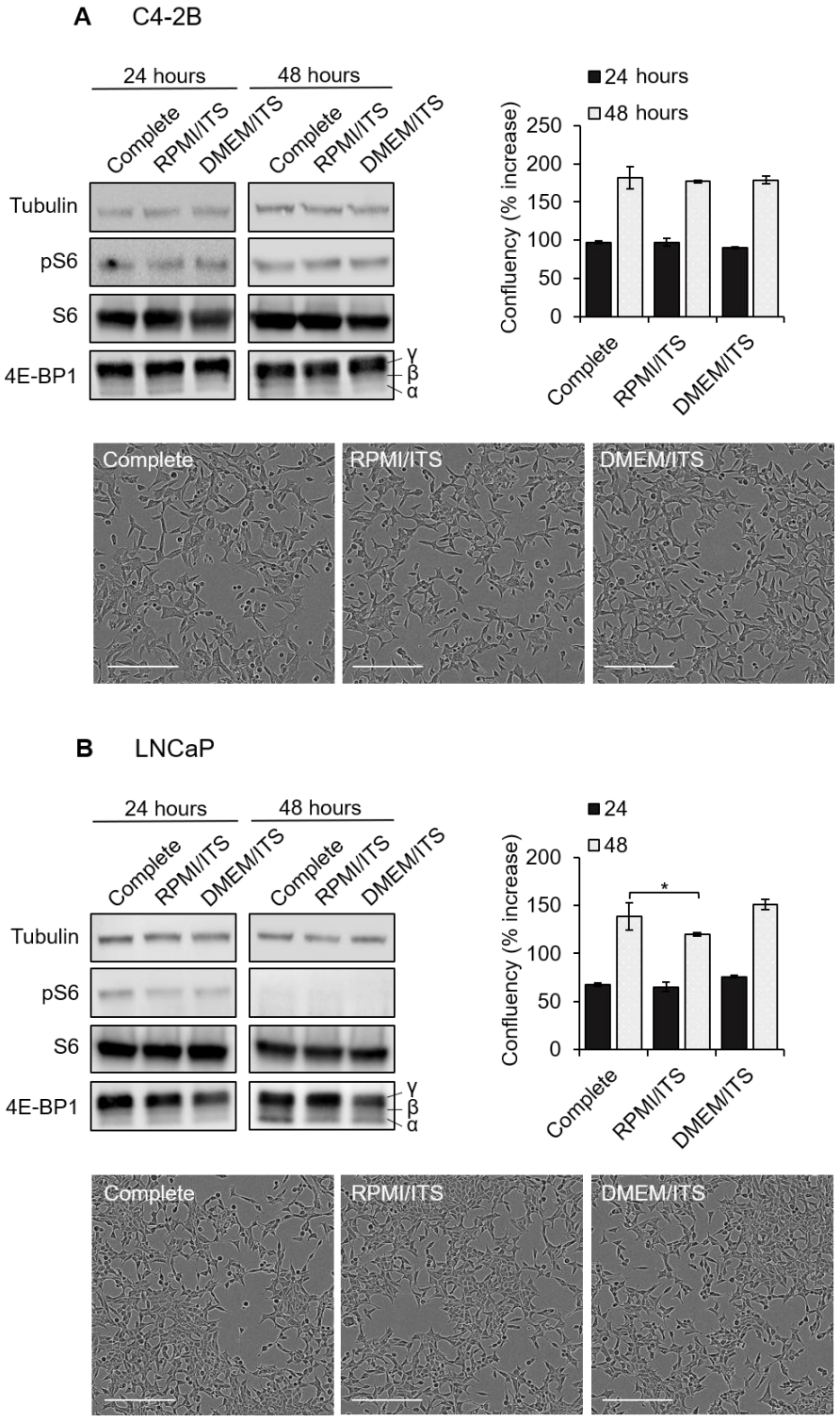


Figure 4.4. Establishing media conditions for EV isolation from LNCaP and C4-2B cells. Cells were cultured in the indicated media for 24 hours before the effects on mTORC1 signalling (A & B, upper left panels), cell growth (A & B, upper right panels) and cell morphology (A & B, lower panel) were assessed. To monitor cell growth, phase-contrast images were collected every two hours using the IncuCyte ZOOM™. Cell confluency was calculated using the IncuCyte™ software and is represented as the fold change in percentage confluency of between 0 and 24 or 48 hours. Results are mean ±S.D of three experiments (n = 8 per condition). ***P < 0.001 vs. control.

in either LNCaP or C4-2B cells after 24 hours (Figure 4.4A and 4.4B, upper left panels). Furthermore, no difference in growth was observed between cells cultured in any of these conditions at this time point either (Figure 4.4A and 4.4B, upper right panels). However, after 48 hours, LNCaP cell growth was reduced by approximately 15% when cultured in basal media with ITS, compared to cells grown in complete medium, whereas no change was observed in C4-2B cell growth. LNCaP and C4-2B cell morphology was also unaffected by either condition after 24 hours (Figure 4.4A and 4.4B, lower panels). Therefore, either RPMI or DMEM/F12 medium seemed suitable to isolate EVs from both cell lines after 24 hours, whereas DMEM/F12 was preferable for isolating EVs from LNCaP cells after 48 hours. Given these findings, I decided to culture both cell lines in RPMI/ITS and restrict the EV isolation period to 24 hours. I reasoned that it would be best to limit the variables introduced during the isolation period, hence the decision to use the standard RPMI culture medium, and that a shorter isolation period would also be preferable. Following these results, I then went on to investigate whether EVs could be isolated from the two cell lines cultured in these conditions.

4.2.5 Characterising PCa EVs isolated via size exclusion chromatography (SEC)

Currently, most studies use ultracentrifugation (UC) as the preferred method to purify EVs. Indeed, UC has long been the gold standard for EV isolation, however I have found that, in my hands, this technique often produces variable results. Reasons for this variability seem to be due to alterations in EV yield as a consequence of human error when resuspending the EV pellet. Indeed, such operator-dependent variability is widely regarded as a common challenge of the UC technique (Momen-Heravi et al, 2013). Additional limitations of UC that have been reported by other users include altered EV morphology and increased EV aggregation, both of which can affect EV function. Therefore, in an attempt to overcome these issues, I decided to use size exclusion chromatography (SEC) as an alternative method to purify EVs (Böing et al., 2014*)*. Since SEC does not rely on high-speed centrifugation, EV structure and function are reportedly

unaffected by this technique (Benedikter et al., 2017). Similarly, SEC requires minimal intervention by the operator, thus opportunities for human error are also limited. Using this technique, EVs secreted from LNCaP and C4-2B cells were isolated from conditioned cell culture medium and characterised according to vesicle size, morphology and protein cargo.

Initially, immunoblotting was performed to determine the purity of the EV sample by comparing the enrichment of commonly used EV protein markers in vesicles isolated using SEC, with whole cell lysate. Since no consensus has yet been reached as to which markers are specific to EVs, a collection of proteins, mostly endocytic in origin and previously identified in vesicles, were used to confirm EV isolation and purity. For example, tetraspanins are a family of membrane proteins abundant within various types of endocytic and EV membranes. Encouragingly, in this analysis, the tetraspanins CD81 and CD63 were highly enriched in EVs isolated from both LNCaP and C4-2B cells using SEC (Figure 4.5A). Similarly, several proteins reportedly involved in MVE and EV biogenesis including Tsg101, Alix and syntenin-1 were also observed in EVs purified from both cell lines, as was the lipid raft-associated protein Flotillin-2. Importantly however, Calnexin, a membrane protein located in the ER, and EEA1, a protein found on early but not late endosomes, were absent from the EV preparations, indicating that the samples were devoid of contaminating vesicles from other non-EV producing compartments. In addition, LAMP-1, a late endosomal marker, was only faintly apparent in the EV samples, highlighting that select protein cargo are incorporated into vesicles released from these compartments.

To further confirm that EVs had been successfully purified from LNCaP and C4-2B cells using SEC, the size distribution and morphology of the isolated vesicles were evaluated by NTA and transmission electron microscopy (TEM) in collaboration with Dr Errin Johnson (Dunn School, Oxford). In terms of size, the NTA profiles appeared similar for EVs isolated from both PCa cell lines; showing a major peak of abundant vesicles around

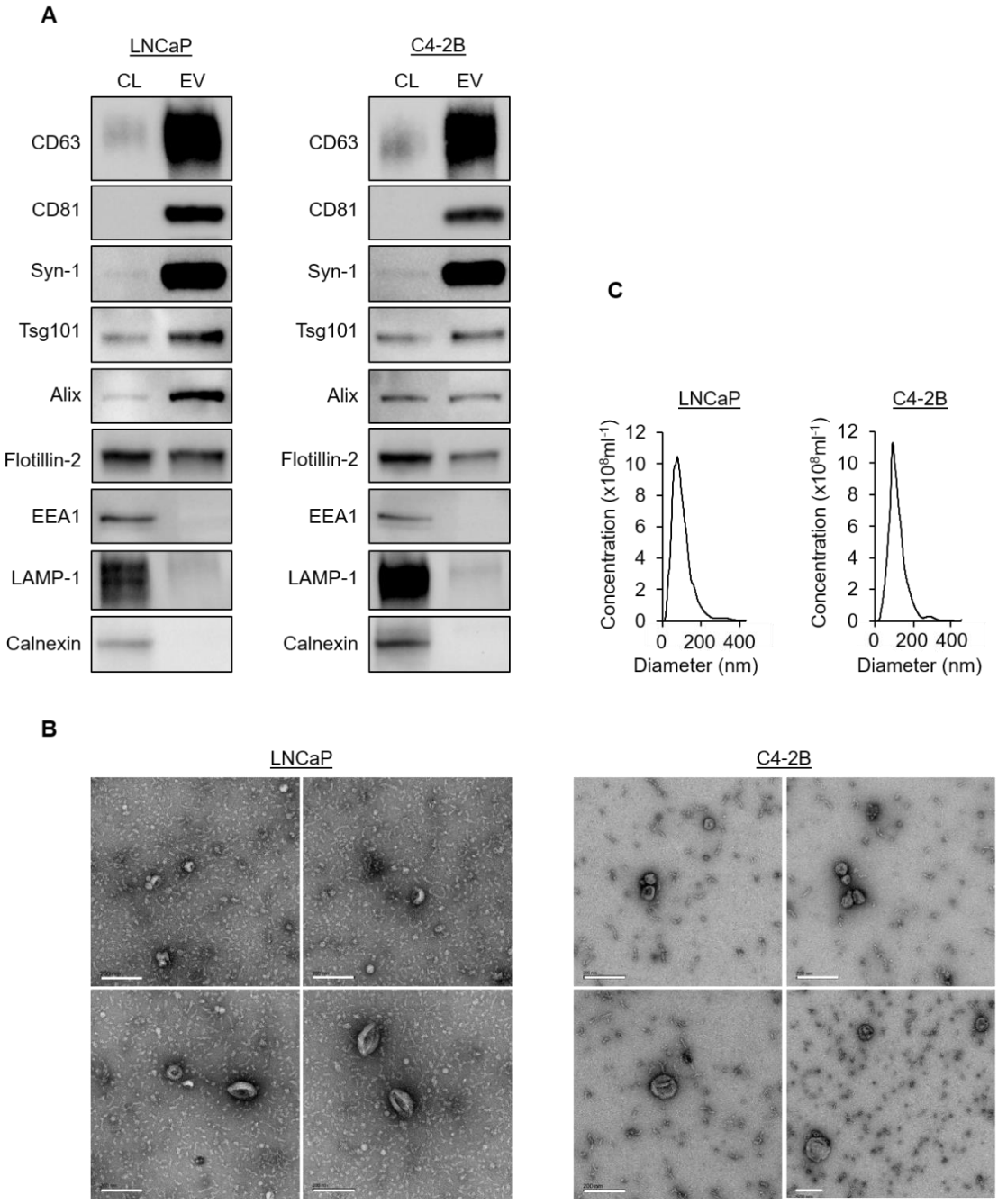


Figure 4.5. Characterisation of EVs isolated from LNCaP and C4-2B cells. EVs were isolated from both cell lines after 24 hours using SEC. **(A)** EV protein and cell lysate (3.5 µg) were analysed via western blotting for several exosome-specific and -non-specific markers. EV morphology and size were examined via TEM **(B)** and NTA **(C)**, respectively. (Scale bar = 200 nm).

110 nm to 120 nm (Figure 4.5C); within the size range reported for exosomes, suggesting that the sample prep may have been enriched for this particular EV population. Moreover, very few particles were found to exceed 200 nm, suggesting that the filtration step

performed prior to SEC was effective and, as a result, the sample was devoid of larger, contaminating vesicles such as liposomes or those shed from the plasma membrane.

Correspondingly, TEM analysis revealed that the conditioned medium taken from LNCaP and C4-2B cells and purified by SEC contained a heterogeneous mixture of vesicles, many of which appeared cup-shaped and measured between 30–100 nm (Figure 4.5B), consistent with the classical morphology of exosomes detailed in the literature and the NTA results reported here. In addition, the microscopy images showed very few contaminant particles in the preparations, further highlighting the purity of the vesicles isolated using this technique. Taken together, the molecular and bio-physical data suggested that SEC was an effective technique to isolate EVs from LNCaP and C4-2B cell culture medium. After confirming the efficacy of the isolation method and characterising the EVs secreted from these two PCa cell lines, I then decided to investigate whether the PAT4-mTORC1 signalling axis regulated the protein profile of EVs released from these cells.

4.2.6 Glutamine starvation induces EV switching in LNCaP cells

As discussed earlier, previous work in the Goberdhan lab has demonstrated that decreased PAT4 expression and/or inhibition of mTORC1 signalling can drive changes in the type of vesicles released from colorectal cancer cells. This phenomenon, termed exosome switching, is characterised by a reduction in the secretion of exosomes from classical MVBs formed from late endosomes and an increase in those released from alternative compartments marked by the small GTPase Rab11. Experimentally, the initial approach I used to try and emulate the effects of decreased PAT4 expression was to deplete the PCa cell culture medium of glutamine. Indeed, it has previously been shown that PAT4 is a glutamine-sensitive amino acid transporter (Fan et al., 2016), therefore I reasoned that starving the cells of glutamine may inhibit PAT4 function and potentially induce similar effects to reducing total PAT4 protein. In addition, increased uptake and metabolism of glutamine has been shown to drive mTORC1-dependent PCa cell growth and is also

associated with disease progression and CRPC (Dasgupta et al., 2015). Hence, glutamine-depletion may be a common microenvironmental stress that accompanies rapid proliferation of aggressive tumour cells and regulates mTORC1 signalling in PCa. Therefore, reducing the levels of extracellular glutamine seemed a particularly relevant condition to examine in the context of both androgen-dependent and -independent PCa.

To begin, I decided to assess whether LNCaP and C4-2B cells were sensitive to glutamine depletion. Both cell lines were cultured in the commercially available, glutamine-free DMEM/F12 medium plus 1% ITS, and a dose-response analysis was performed to assess the effects of supplementing the medium with different concentrations of glutamine on mTORC1 activity after 24 hours. In LNCaP cells, phosphorylation of S6 and 4E-BP1 both appeared sensitive to changes in extracellular glutamine (Figure 4.6A, upper left panel). For instance, levels of p-S6 were almost completely lost when the concentration of glutamine was reduced to 25% of control, whereas reductions in hyperphosphorylated 4E-BP1 were seen once the glutamine concentration dropped below 2% of control levels. Further analysis revealed that, in both cell lines, p-S6 and hyperphosphorylated 4E-BP1 were sensitive and resistant to rapamycin treatment, respectively; indicating that glutamine starvation likely affects both rapamycin-resistant and -sensitive forms of mTORC1 in LNCaP cells. Conversely, no difference in phosphorylation of either of these two targets was detected in C4-2B cells cultured in the absence of glutamine for 24 hours. Hence, C4-2B cells appeared less sensitive to short-term glutamine depletion, at least in terms of mTORC1 activity. No difference in mTORC2 activity (i.e. p-Akt) was observed following glutamine depletion. Interestingly, I also noted that PAT4 protein levels were increased in C4-2B cells compared to LNCaP cells (Figure 4.6B), raising the intriguing possibility that enhanced PAT4 expression may underpin the glutamine insensitivity displayed by C4-2B cells. Unfortunately however, due to time limitations, I was unable to explore this theory further.

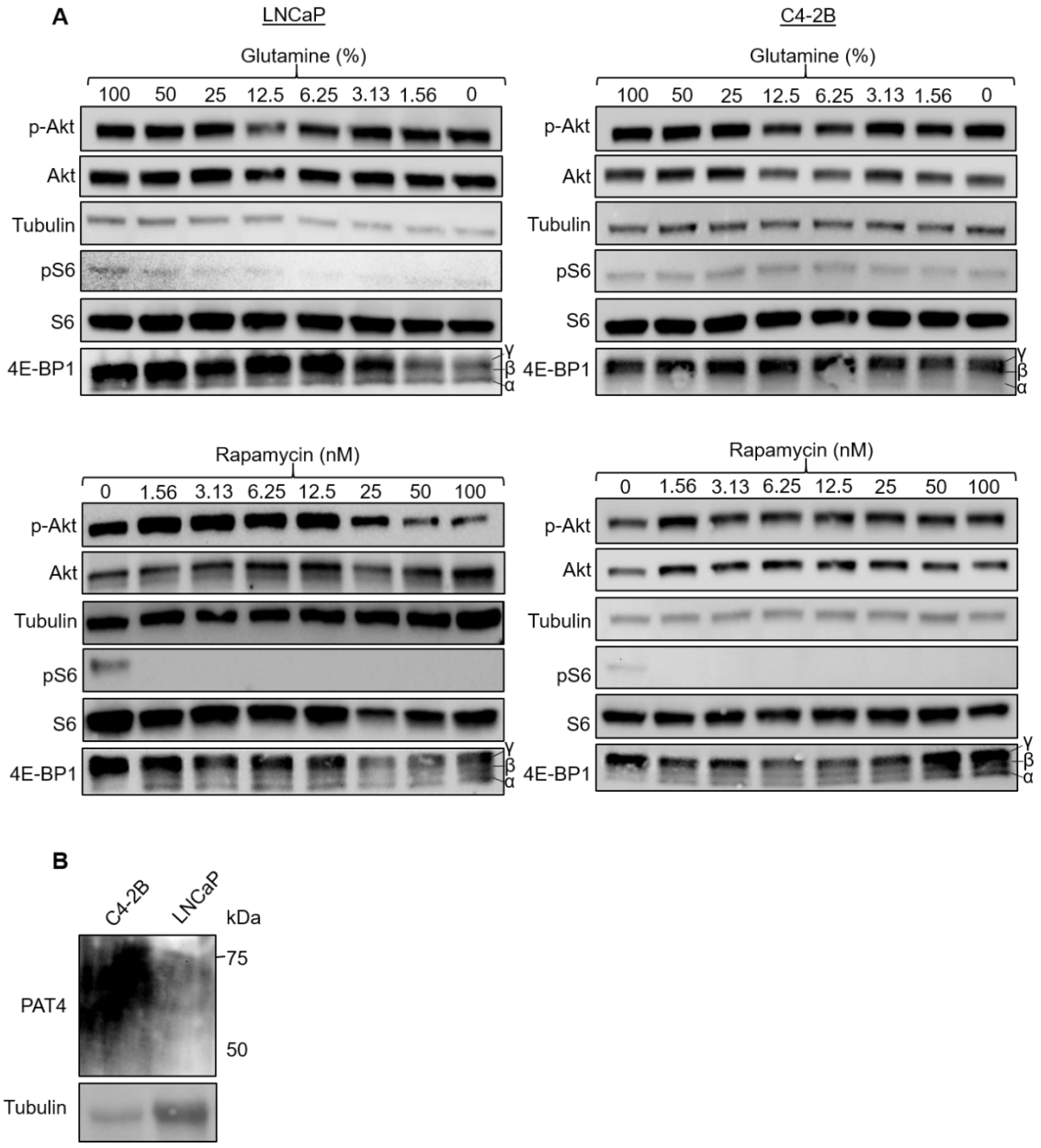


Figure 4.6. Examining the sensitivity of LNCaP and C4-2B cells to glutamine-depletion and rapamycin treatment. Western blot showing the dose-response of mTORC1 signalling in each cell line to glutamine depletion (**A**, upper panels) and rapamycin treatment (**A**, lower panels) after 24 hours. **(B)** The level of PAT4 protein in each cell line.

Following these results, I then went on to investigate whether glutamine starvation could influence the type of EVs secreted from both PCa cell types. I reasoned that, by analysing two such closely related cell lines with different sensitivities to glutamine depletion, I would be better positioned to identify the regulatory mechanisms responsible for modulating changes in EV signalling in response to environmental stress in PCa.

Regarding the conditions for EV isolation, cells were cultured in glutamine-free DMEM/F12 medium plus 1% ITS, supplemented with either the concentration of glutamine found in standard DMEM/F12 medium (i.e. 2 mM) for the control, or 1% of this for the depleted condition (i.e. 0.02 mM). The latter concentration was chosen as it represented the concentration of glutamine in which the level of 4E-BP1 phosphorylation was reduced the most in LNCaP cells, and hence, the situation in which signalling via the PAT4-mTORC1 axis would presumably be at its lowest. LNCaP and C4-2B cells were cultured in these conditions for 24 hours before EVs were isolated using SEC. To confirm that both cell lines were responding appropriately to treatment, cell lysates were analysed by western blotting to assess mTORC1 activity. Consistent with the dose-response data, compared to LNCaP cells cultured in media supplemented with the standard concentration of glutamine, cells maintained in glutamine-depleted conditions showed reduced levels of both S6 and 4E-BP1 phosphorylation (Figure 4.7A, right panel), confirming the treatment conditions were effective. Likewise, only a very subtle change in mTORC1 signalling was observed in C4-2B cells cultured in the same conditions (Figure 4.7B, right panel).

In addition to examining changes in mTORC1 activity, the expression of several widely-used EV protein markers, alongside Rab11, were also examined in the EV-secreting cells. This particular analysis was important to determine whether total protein levels were altered following glutamine depletion, as this could affect the relative abundance of these proteins in EVs and confound the results. Encouragingly, none of these markers were changed significantly in either cell line following glutamine starvation. Following these results, I then went on to look for changes in the protein profiles of the secreted EVs.

However, examining EV proteins by western blotting raises several issues, namely the question of how to achieve equal protein loading. Unlike analysing protein levels in cell lysate samples where housekeeping genes are used to control for total protein amount, no

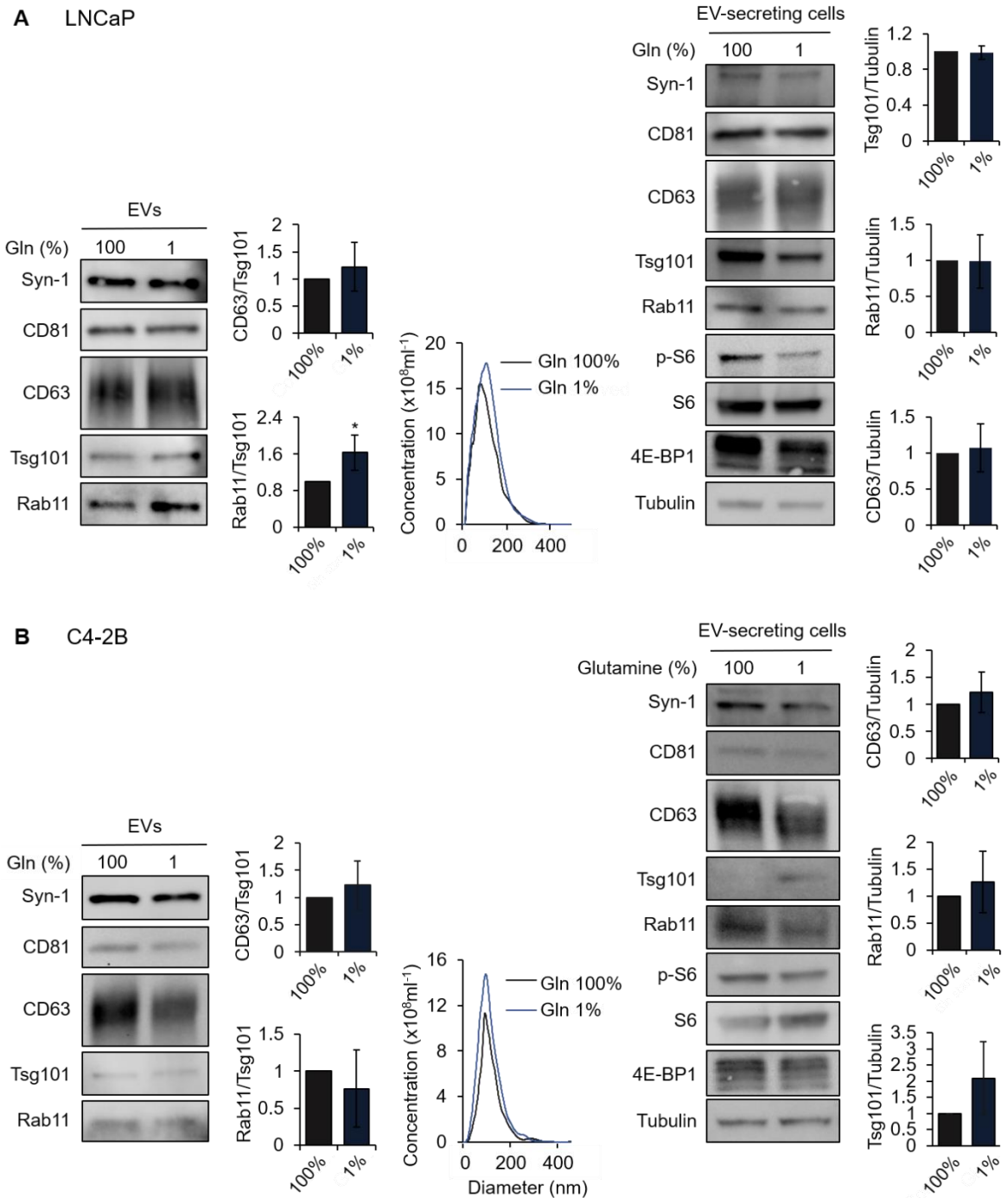


Figure 4.7. Glutamine-depletion alters EV signalling in LNCaP, but not C4-2B, cells. (A & B, **right** panels) Cells were cultured in control (100% Gln) or nutrient depleted conditions (1% Gln) for 24 hours before the effects on mTORC1 activity and the expression of several canonical exosome proteins were examined in the cell lysate via western blotting. EVs were isolated from these cells after the same period and characterised for changes in the level of cargo proteins via western blotting (A & B **left** panels) and size via NTA (A & B, **middle** panels). Results are mean ±S.D of three independent experiments (1 replicate/ condition). *P < 0.05 and **P < 0.01 vs. control.

such protein common to, and consistent within, all EVs has yet been identified. Hence, comparative EV analysis can be challenging. Moreover, it is unknown whether glutamine depletion affects EV number or protein content, therefore using NTA or BCA quantification methods for normalisation, as is common in other studies, seemed unsuitable for this work. In addition, cell growth is also likely affected by this treatment. Therefore, following the advice of Dr Shih-Jung Fan (post-doc, Goberdhan lab), I decided to characterise changes in EV secretion on a cell-by-cell basis by normalising the volume of EV sample loaded on the gel to the total protein content of the cells. Hence, any alterations observed in EV cargo should signify a change in EV regulation, not cell number.

Among the proteins examined, Tsg101 was the marker most consistently unchanged in EVs following glutamine depletion. Therefore, the level of Tsg101 was used as a control to which other EV cargos were compared, and their relative abundance determined. In terms of the EVs isolated from LNCaP cells, additional proteins that were unaffected by glutamine depletion included syntenin-1 and CD81 (Figure 4.7A, right left panels). Surprisingly, CD63, a protein characteristically reduced in EVs secreted from glutamine-depleted HCT116 cells, was also unchanged. Hence, it appeared that decreased levels of glutamine could differentially affect EV secretion from different cancer cell lines. On the other hand, Rab11 was significantly increased in EVs following glutamine depletion, consistent with the exosome switch phenotype as characterised in colorectal cancer cells. Therefore, parallels seemingly do exist between different cancer cell lines, although some aspects of this regulation may be cell type-specific. Analysis of these EV preparations with NTA further revealed no difference in vesicle size or number, suggesting that cells cultured in glutamine-deprived conditions secrete a similar of EVs to control cells, but enriched for a different set of cargoes (Figure 4.7A, middle panel).

Interestingly, no change was observed in either Rab11 or CD63 or the number of EVs secreted from C4-2B cells following glutamine depletion (Figure 4.7B). This finding is

consistent with these cells being insensitive to glutamine starvation and mTORC1 signalling being the regulatory mechanism responsible for the change in EV cargo observed in LNCaP cells. In order to explore this theory further, I decided to examine the effects of inhibiting mTORC1 activity directly on EV signalling in both cell lines.

4.2.7 mTORC1 inhibition induces EV switching in LNCaP and C4-2B cells

To assess the effects of mTORC1 inhibition on EV signalling in LNCaP and C4-2B cells, a drug screen was initially conducted to determine the concentration of inhibitor to use for this analysis. Torin1 is an ATP-competitive inhibitor of mTOR that is widely used in mTOR research. I had previously found Torin1 to be a particularly potent inhibitor of mTORC1 activity in several other cancer cell lines (data not shown), and therefore decided to use the agent again for this analysis. Like other second generation mTOR inhibitors, Torin1 inhibits the activation of all mTOR-containing complexes, including rapamycin-sensitive and -insensitive mTORC1, and mTORC2. Given my earlier findings hinted that PAT4 may promote rapamycin-resistant mTORC1 activity in VCaP cells, I reasoned that such an agent may effectively block this signalling axis in LNCaP and C4-2B cells, enabling the role of this pathway in regulating EV secretion to be examined.

Experimentally, both cell lines were cultured in RPMI medium plus 1% ITS and a dose-response analysis was performed by supplementing the medium with several different concentrations of Torin1 for 24 hours (Figure 4.8A and 4.8B). Overall, treatment with Torin1 completely blocked mTORC1 signalling in LNCaP and C4-2B cells, as evidenced by the total loss of p-S6 and hyperphosphorylated 4E-BP1 at high concentrations of inhibitor. Specifically, for LNCaP and C4-2B cells, I chose 120 nM and 160 nM Torin1 as the respective drug concentrations to be used for further analysis, as each represented the lowest concentration of drug in which p-S6 and hyperphosphorylated 4E-BP1 were completely lost. Although mTORC2 was also inhibited by the treatment, the p-Akt signal

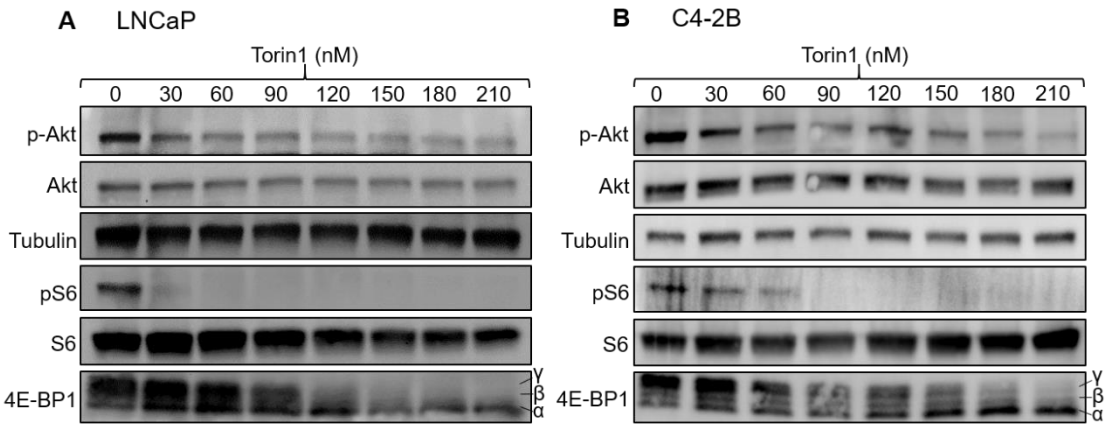


Figure 4.8. Assessing the sensitivity of LNCaP and C4-2B cells to Torin1 treatment. Western blot showing the dose-response of mTORC1 signalling in LNCaP **(A)** and C4-2B cells **(B)** to Torin1 treatment after 24 hours.

was still detectable when the phosphorylation of 4E-BP1 and S6 had diminished, suggesting that Torin1 may exhibit greater selectivity towards mTORC1 in these cells.

For EV isolation, cells were cultured in RPMI medium supplemented with 1% ITS and treated with either vehicle (DMSO) or Torin1 for 24 hours, after which vesicles were isolated using SEC. As with glutamine depletion, no change in EV number or size was observed in either cell line following Torin1 treatment (Figure 4.9A and 4.9B, middle panels). In addition, cell lysate analysis showed that mTORC1 signalling was effectively reduced by Torin1 in both cell lines after 24 hours, whereas no significant differences were observed in the expression levels of any of the EV protein markers examined (Figure 4.9A and 4.9B, right panels). In terms of EV cargo, in LNCaP cells, the effects of Torin1 treatment were similar to those observed following glutamine depletion. That is, there was an increase in Rab11 yet no significant change in syntenin-1 (Figure 4.9A, left panel), indicating that the changes in EV signalling induced by glutamine depletion may be mTORC1- dependent. Indeed, the increase in Rab11 following Torin1 treatment was approximately two-fold greater than the increase observed following glutamine starvation. Furthermore, in contrast to the effects of

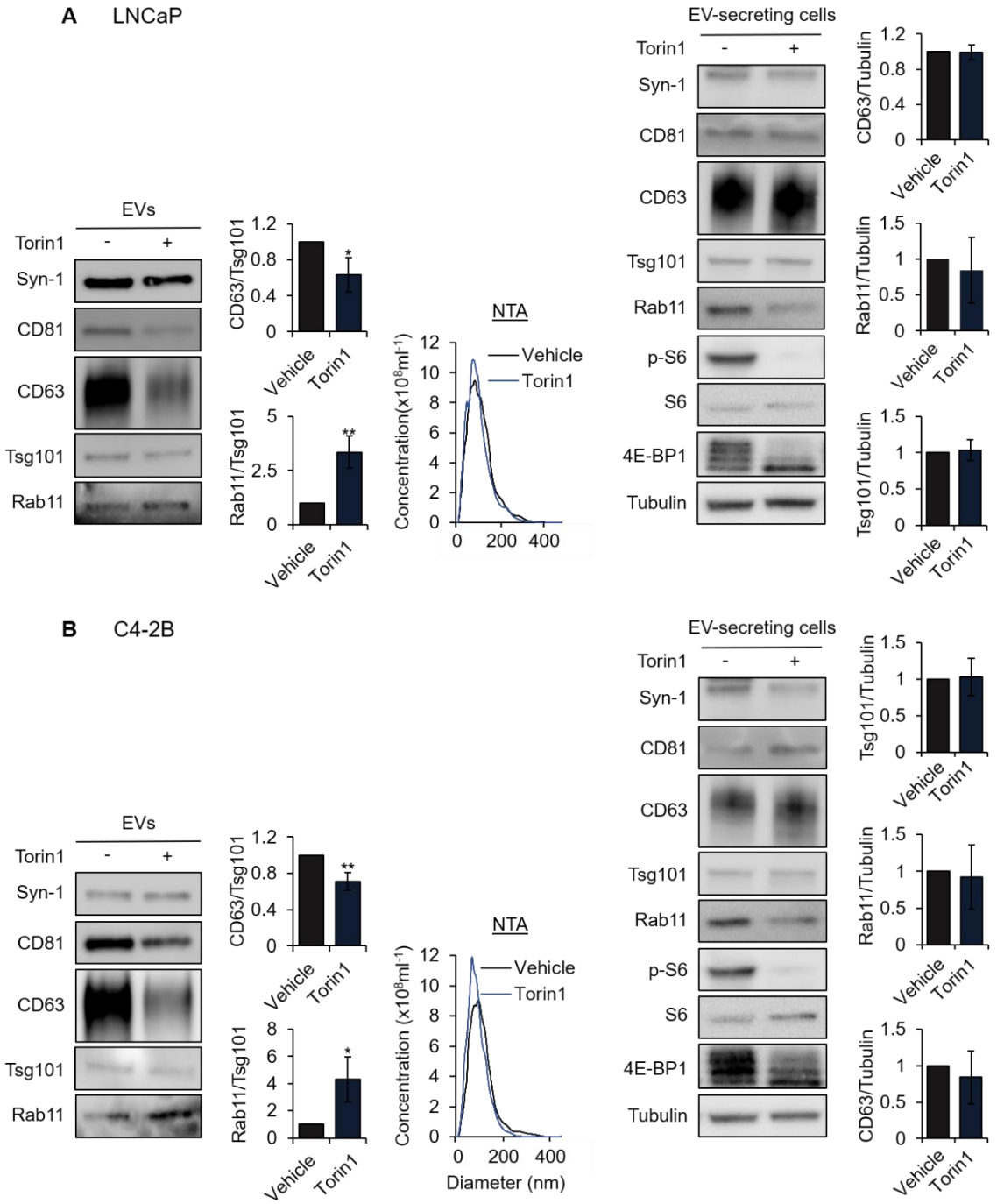


Figure 4.9. mTORC1 inhibition alters EV signalling in both LNCaP and C4-2B cells. (A & B, **right** panels) Cells were treated with vehicle (DMSO) or the mTOR inhibitor Torin1 (LNCaP; 120 nM, C4-2B; 160 nM) for 24 hours before the effects on mTORC1 activity and the expression of several canonical exosome proteins were examined in the cell lysate via western blotting. EVs were isolated from these cells after the same period and characterised for changes in the level of cargo proteins via western blotting (A & B **left** panels) and size via NTA (A & B, **middle** panels). Results are mean ±S.D of three independent experiments (n = 1 per condition). *P < 0.05 and **P < 0.01 vs. control.

glutamine depletion, a significant decrease in CD63 in EVs was also observed following Torin1 treatment, consistent with the exosome switch phenotype as described in HCT116 cells. Considering Torin1 induced a greater reduction in both S6 and 4E-BP1 phosphorylation, these results suggest that the degree of mTORC1 inhibition may correlate with the magnitude of the switching phenotype. In addition, in C4-2B cells, increased secretion of Rab11-positive EVs and decreased secretion of CD63-positive EVs was also observed following Torin1 treatment (Figure 4.9B, left panel). Given that such changes were absent in the EVs isolated from these cells when cultured in glutamine-depleted conditions, where the phosphorylation of S6 and 4E-BP1 were unaffected, these findings further point towards mTORC1 as the signalling pathway responsible for regulating these changes in EV cargo.

4.3. Discussion

The PAT4-mTORC1 signalling axis has recently been revealed as an important regulator of EV secretion in colorectal cancer cells. Inhibition of this pathway in HCT116 cells was shown to promote the release of a novel EV subtype that can stimulate pro-tumorigenic effects in recipient cancer cells. In PCa, accumulating evidence has implicated a role for EVs in disease progression (Pan et al, 2017). In addition, many components of the mTORC1 pathway are frequently altered in PCa, including PAT4. Hence, if such a regulatory axis exists in PCa cells, then the clinical implications of targeting this pathway could be far-reaching. In this chapter, I set out to understand the role of PAT4 in PCa by characterising the effects of knocking down this transporter on mTORC1 activation and PCa cell growth. I also employed pharmacological mTORC1 inhibitors and glutamine depletion to assess whether inhibiting PAT4 signalling could affect EV secretion.

Overall, I found that PAT4 expression is up-regulated following androgen treatment in VCaP cells, and that, functionally, PAT4 is involved in mediating the effects of androgens on mTORC1 signalling and PCa cell growth, at least in part. Moreover, I showed that inhibiting PAT4 signalling, via glutamine depletion, increased the secretion of Rab11-positive EVs from PCa cells that were sensitive to glutamine starvation, and that this effect could be replicated using a small molecule inhibitor of mTORC1. Overall, these findings indicate that mTORC1 signalling regulates EV secretion in PCa cells, and therefore, that alterations in this pathway caused by genetic mutation, pharmacological inhibition or nutrient deprivation, may have important effects on the type of EVs released into the TME.

4.3.1 Androgens promote PAT4 expression

My analysis in VCaP cells revealed that PAT4 mRNA and protein levels are upregulated in response to DHT treatment. Whether the PAT4 gene is a direct target of AR-mediated transcription or is indirectly affected was not determined in this analysis, although future

studies could explore this further by examining the PAT4 promoter region for evidence of AR binding sites. Overall, my findings could offer a potential explanation as to why PAT4 levels are increased in malignant prostate tissue compared to benign tissue, as has been reported (Dan Stevens; Figure 1.8). Indeed, the critical role that androgens play in regulating the initial growth of PCa cells as well as disease progression has been well established (Lin et al., 1998; Abate-Shen et al., 2000; Nantermet et al., 2004); hence, it is possible that the upregulation of PAT4 protein observed in PCa tissue reflects increased AR signalling in the corresponding PCa cells. Further analyses to examine the expression of additional AR regulated gene targets would be useful to determine whether AR activity is indeed increased in these cells and correlates with PAT4 levels. Supporting this notion, expression of the well-established AR gene target TMPRSS2 was shown to be increased in PCa cells compared to non-transformed cells from the same prostate tissue sample (Chen et al., 2010). Likewise, levels of FKBP51, a newly-identified AR gene target, were also found to be higher in PCa tumours than in benign prostate tumours (Velasco et al., 2004). These findings indicate that expression of AR-regulated genes is typically enhanced in PCa, consistent with PAT4 being a valid AR target.

Given these findings, it would be interesting to examine PAT4 levels in PCa tissue or cells derived from patients with advanced and metastatic disease to investigate whether PAT4 levels correlate with disease progression. Indeed, the expression of other known AR target genes are altered as PCa advances and AR signalling becomes aberrant. For instance, PSA, the best-characterised AR-regulated gene, is used as a readout for AR signalling to monitor BCR in patients following androgen ablation. Hence, it follows that the expression of other androgen-regulated genes, i.e. PAT4, may be similarly affected in patients undergoing such treatment and in those who become resistant to ADT. Indeed, the expression of PAT4 in response to bicalutamide treatment was only examined briefly during this project, however preliminary results suggested that blocking AR signalling could reduce PAT4 protein levels in VCaP cells (data not shown). Intriguingly, reductions in *PAT4* mRNA

are significantly more common in patients with metastatic than localised PCa (Taylor et al., 2010). Hence, one possibility could be that those patients with metastatic disease had received ADT, resulting in decreased PAT4 expression. Nonetheless, a more complete analysis is required to fully characterise the effects of anti-androgen treatment on the regulation of PAT4 in PCa. Ultimately, determining whether the expression profile of PAT4 changes as PCa progresses, and if these changes correlate with patient outcomes, seems to me an important clinical aim for future studies, especially given the potential role I have identified for PAT4 in regulating cell growth and EV signalling.

However, it should also be restated that, whilst I found that androgens could increase PAT4 expression in VCaP cells, this effect was not observed in LNCaP cells. One major difference between LNCaP and VCaP cells is the status of PTEN; with *PTEN* being wild type in VCaP cells and null in LNCaP cells (Vlietstra et al.,1998). Hence, one possible explanation for the difference in my findings could be that, in LNCaP cells, mTORC1 signalling is constitutively active due to PTEN loss, and therefore, these cells do not require increased PAT4 to promote mTORC1 activity and mediate androgen-dependent growth. Assessing this response in additional PCa cell lines with different PTEN status' will be necessary to explore this theory further.

4.3.2 DHT-induced mTORC1 activation and cell growth is partially mediated by PAT4

In terms of function, I have shown that AR signalling drives mTORC1 activation and VCaP cell growth, in part, via PAT4-dependent mechanisms. These results are consistent with the functional effects reported for PAT4 in HCT116 colorectal cancer cells and provide a possible explanation as to why androgens promote PAT4 gene expression: to augment PCa cell growth. Certainly, it makes sense that as a critical mediator of PCa cell growth, the AR signalling pathway would regulate the expression of genes involved in crosstalk with the master regulator of cell growth, mTORC1. Indeed, this positive regulation of mTORC1 signalling by androgens has been previously established (Xu et al., 2006; Rai et al., 2010;

Wu et al., 2010; Tsouko et al., 2014). Furthermore, findings from these studies as well as others have suggested that a key way in which AR signalling modulates mTORC1 activity is by increasing the expression of proteins that enhance nutrient availability and cellular metabolism (Waghray et al., 2001; Nelson et al., 2002; Deprimo et al., 2002; Velasco et al., 2004; Shaffi et al., 2012). For instance, androgen treatment reportedly increases the expression of the aspartate transporter EAAC1 in prostate cells (Lao et al., 1993) and the expression of LAT1 and LAT3, two L-Type amino acid transporters, in PCa cells (Wang et al., 2011). Moreover, several transporters belonging to the SLC family of proteins are also upregulated by androgens (Xu et al., 2006) and have similarly been shown to mediate the effects of androgens on PCa cell growth by enhancing mTORC1 activity (White et al., 2017). Such findings are consistent with the results presented in this chapter and the proposed role for PAT4 as a regulator of mTORC1 signalling and PCa cell growth. Nonetheless, although I have shown that knocking down PAT4 reduces the stimulatory effects of androgens on PCa cell growth and mTORC1 activity, and that inhibition of mTORC1 also diminishes these responses, I have yet to show that PAT4 mediates its effects on PCa cell growth via mTORC1. Indeed, it is possible that other pathways also affected by decreased PAT4 expression are responsible for the observed effects. Therefore, additional studies are necessary to clarify this issue. For instance, inhibition of mTORC1 and PAT4 knockdown could be assessed together.

Nonetheless, whether PAT4 acts via mTORC1 to mediate the effects of androgens on VCaP cell growth, this work still highlights an important finding: PAT4 serves to regulate mTORC1 signalling in PCa cells. Although previous studies have demonstrated that androgens can modulate mTORC1 activity by promoting the expression of amino acid transporters and enhancing nutrient uptake into the cell (Xu et al., 2006; Wang et al., 2011), no role has yet been proposed for these transporters in amino acid sensing. However, it seems reasonable to assume that, as well as increasing the amount of nutrients available to a cell, androgens may also enhance the cells ability to relay this information to the

regulatory components responsible for modulating cell growth. Indeed, the observation that PAT4 is primarily located intracellularly in PCa tissue (Figure 1.8) suggests that this protein does not regulate mTORC1 by increasing amino acid uptake into the cell, supporting the notion that PAT4 may instead function as an amino acid sensor. However, the fact that PAT4 mainly localises to plasma membrane in benign prostate tissue samples suggests that the role of PAT4 may change following the development of PCa, perhaps reflecting the altered metabolic demands of the growing tumour. This observation could also reveal an underlying regulatory mechanism controlling PAT4 activity. For instance, work completed in *Drosophila* has shown that elevated PI3K/Akt/Rheb signalling synergistically enhances the growth-promoting effects of the fly PATs via a mechanism involving the translocation of the PATs to intracellular compartments (Ögmundsdóttir et al., 2012). Considering mutations that lead to the constitutive activation of the PI3K/AKT/mTOR pathway have been reported in 42% of primary and 100% of metastatic PCa cases (Taylor et al., 2010), it would be interesting to determine whether similar alterations in signalling underpins the cytoplasmic localisation of PAT4 in PCa tissue. Intriguingly, a similar change in the spatial distribution of p-mTOR has been reported, with higher levels observed in the cytoplasm in malignant prostate tissue compared to benign samples, where p-mTOR is primarily located at the plasma membrane (Kremer et al., 2006). Hence, one could speculate that the subcellular localisation of PAT4 and p-mTOR are similarly regulated to ensure the successful activation of mTORC1. Nonetheless, the intracellular distribution of PAT4 was not examined in this analysis, hence it is possible that in VCaP cells PAT4 is located at the plasma membrane.

4.3.3 SEC is an effective method to isolate EVs from PCa cell culture media

Another aim of this chapter was to establish a set of conditions to examine the effects of mTORC1 inhibition on EV secretion from PCa cell lines. The parameters I set to realise this aim included; identifying culture conditions that were both serum-free and capable of maintaining mTORC1 signalling during the period of EV collection. The absence of serum was particularly important to ensure foreign EVs did not contaminate the sample

preparations, therefore a cocktail of ITS was added to the media instead. Ensuring that the cells did not become growth factor-depleted was also essential to sustain mTORC1 activity, support cell growth and, ultimately, to determine the consequences of inhibiting mTORC1 on EV secretion. Initially, I examined the effects of culturing LNCaP and C4-2B cells in serum-free culture media on cell growth and mTORC1 activity. Based upon this analysis, after 24 hours incubation, mTORC1 signalling and PCa cell growth and morphology appeared similar in the cells cultured in serum-free conditions to those grown in complete growth media. Therefore, EVs were isolated from LNCaP and C4-2B cells cultured in their standard basal media supplemented with ITS.

In terms of the isolation technique, I had previously encountered reproducibility and yield issues with UC, the most commonly used method for EV purification. Therefore, instead, I decided to use SEC, an emerging EV purification technique that has been used successfully to isolate vesicles from numerous biological fluids, including sera, ascites and saliva (Taylor et al., 2011). Moreover, this technique requires very little handling by the operator, therefore issues with reproducibility are rare and EV recovery is reportedly high (Nordin et al., 2015). Using SEC, I found that EVs isolated from LNCaP and C4-2B cell culture media were enriched for numerous exosome markers, including the tetraspanins CD63 and CD81 and several proteins involved in MVE and exosome biogenesis. Importantly, proteins considered to be markers of contamination were absent, highlighting the purity of this isolation method. In addition, the majority of particles recovered using SEC measured within the exosomal size range and appeared cup-shape in morphology; both observations supporting the notion that a large proportion of these EVs may be exosomes. Nonetheless, the possibility that vesicles other that exosomes are present in these preparations cannot be eliminated, hence the reason why the isolates are referred to as EVs. Taken together, these data indicate SEC to be an effective technique to isolate EVs from LNCaP and C4-2B cell culture medium.

4.3.4 The mTORC1 pathway regulates EV signalling in PCa cells

In addition to mediating the stimulatory effects of androgens on cell growth and mTORC1 activation, the data presented in this chapter also highlights a potential role for the PAT4-mTORC1 signalling axis in regulating EV signalling in PCa. The strategy I took to assess whether PAT4 was involved in mediating this process was two-fold and included treating LNCaP and C4-2B cells with either Torin1, a pharmacological inhibitor of mTORC1, and depleting the cell culture medium of glutamine. As PAT4 is a glutamine-sensitive transporter and a positive regulator of mTORC1 activity, I reasoned that such manipulations may have comparable effects to blocking PAT4 signalling directly. Interestingly, consistent with the exosome switch phenotype described in the HCT116 cell line following PAT4 knockdown, inhibition of mTORC1 signalling similarly altered the protein profile of EVs secreted from LNCaP and C4-2B cells. This change in EV cargo, stimulated by treatment with Torin1, was characterised by an increase in the proportion of Rab11-positive EVs and a decrease in vesicles marked by CD63, whereas the level of other EV markers such as syntenin-1 and CD81 remained unchanged. Whilst this result does implicate mTORC1 as a key regulator of this event, non-specific effects of Torin1 on other signalling pathways (e.g. mTORC2) cannot be discounted. Nonetheless, glutamine depletion, which had very little effect on mTORC2 activity, also induced the secretion of Rab11-positive vesicles from LNCaP cells, supporting the notion that this effect is indeed mTORC1-specific. However, an alternative interpretation that should not be overlooked is the possibility that Torin1 stimulates the shedding of vesicles from the plasma membrane, perhaps due to enhanced apoptosis. Although vesicle size was unaffected by Torin1 treatment, suggesting no such change in EV identity, cell death should be examined to confirm this.

Interestingly, although glutamine depletion enhanced the secretion of EVs marked with Rab11 from LNCaP cells, no change was observed in EVs secreted from C4-2B cells cultured in the same conditions. One difference I identified between the two cell lines which could potentially explain this finding was their differential sensitivities to glutamine

starvation. Indeed, unlike their glutamine-sensitive counterparts, C4-2B cells were resistant to short-term glutamine starvation, at least in terms of mTORC1 activity, or more specifically, the phosphorylation 4E-BP1 and S6. Considering both cell lines exhibited similar responses to mTORC1 inhibition using Torin1 (i.e. increased secretion of Rab11), these findings suggest that the mTORC1-EV regulatory pathway still exists in C4-2B cells, but that regulators upstream of mTORC1 may have become altered as PCa progresses. Intriguingly, I found that the level of PAT4 protein was greater in C4-2B cells than in LNCaP cells. Considering C4-2B cells reportedly exhibit hyperactive AR signalling, this finding is not unexpected given I have also shown that androgens positively regulate PAT4 expression. Hence, together these observations raise the attractive possibility that as PCa progresses and tumour cells becomes androgen-independent, the expression of PAT4 increases, resulting in enhanced tumour growth and resistance to nutrient deprivation. Nonetheless, further work is required to determine whether increased PAT4 expression does indeed enable cells to overcome glutamine depletion in the TME.

Overall, it appears that mTORC1 signalling regulates a similar exosome switching event in PCa cells to that originally characterised in colorectal cancer cells. Given that I observed this effect using both glutamine depletion and pharmacological inhibition of mTOR, it seems likely that PAT4 will also play a role in regulating EV signalling in PCa. However, further studies (i.e. PAT4 knockdown) are ultimately necessary to confirm this.

4.3.5 Clinical implications

In PCa, PAT4 is upregulated and, as I have shown in PCa cells, acts to positively regulate mTORC1 signalling and growth. In addition, mining of the human PCa dataset (Taylor et al., 2010) has revealed that changes in PAT4 copy number and mRNA expression are significantly more common in metastatic PCa than primary disease. Moreover, changes in PAT4 expression are associated with biochemical and metastatic recurrence following

radical treatment for localised PCa. Altogether, these findings highlight a potential role for PAT4 in PCa progression and provide a rationale for targeting PAT4 therapeutically.

However, as discussed in the introduction (1.3.3.5), activating mutations in the PI3K/AKT/mTOR pathway are highly prevalent in the development and progression of PCa (Stemke-Hale et al., 2008; Taylor et al., 2010; Thorpe et al., 2015), therefore a similar rationale has been previously used to justify targeting other components in this pathway. Generally, this strategy has shown limited clinical success (Reardon et al., 2010; Nakabayashi et al., 2012; Armstrong et al., 2013; Wen et al., 2014; Ma et al., 2015; Statz et al., 2017). One explanation for this concerns the fact that first generation mTOR inhibitors do not block rapamycin-resistant mTORC1 activity (Feldman et al., 2009; Thoreen et al., 2009; Hsieh et al., 2010). However, PAT4 has been identified as a positive regulator of rapamycin-resistant mTORC1 signalling in colorectal cancer cells; a role my findings support for the transporter in PCa cells as well. Hence, it is possible that inhibition of PAT4, in combination with rapamycin, could provide a more complete blockade to mTORC1 signalling in PCa tumours, improving the outcomes for patients with activating mutations in this pathway. Moreover, if proven that PAT4 does indeed regulate this branch of mTORC1 signalling in PCa, it could also represent a new biomarker to identify more aggressive tumours that are rapamycin-resistant, as well as those patients who may respond more favourably to treatment.

Another important reason why mTOR inhibitors may have shown such poor clinical efficacy concerns the complex regulatory network that exists between mTOR and other signalling pathways, and the subsequent activation of these pathways following mTORC1 inhibition. For instance, treatment with various rapalogues can actually enhance PI3K/Akt signalling in cancer cells (Shi et al., 2005), despite inhibition of mTORC1 activity. Mechanistic studies have revealed that such a response is a result of rapalogues blocking the phosphorylation of S6, a phosphorylation event which typically results in mTORC2 inhibition. Consequently,

mTORC2 activity is increased in these cells, enabling cell survival. Moreover, in PCa cells, increased Akt signalling promotes AR activity and disease progression (Wang et al., 2008*).* Given the findings I have presented in this chapter indicate that PAT4 knockdown inhibits 4E-BP1 phosphorylation more than S6, inhibiting PAT4 could potentially induce the same growth-inhibitory effects obtained using mTORC1 inhibitors without increasing AR activity. I have also shown that knocking down PAT4 can block both androgen-dependent and -independent PCa cell growth, thus inhibiting PAT4 function may be a promising therapeutic strategy for the treatment of both hormone naïve and castrate-resistant disease. Overall, my findings propose PAT4 as a potential clinical target for the selective inhibition of specific mTORC1 activities, perhaps as part of a future combinatorial approach to block both PI3K/AKT/mTOR and AR signalling in PCa.

Additional findings presented in this chapter, although still speculative, raise the interesting possibility that PAT4 may also be involved in mediating resistance to glutamine depletion in PCa cells. Indeed, I have shown that androgens increase PAT4 expression in PCa cells and, consistent with this, that PAT4 protein levels are greater in the androgen-independent C4-24 cells versus the androgen-sensitive LNCaP cells. Furthermore, C4-2B cells are resistant to glutamine depletion. Hence, one possible explanation could be that as PCa progresses and tumour cells becomes androgen-independent, PAT4 levels increase, and cells becomes resistant to glutamine deprivation as a result. Indeed, alterations in other amino acid-sensing components of the mTORC1 pathway have also been reported in cancer and proposed to similarly enable tumour cells to overcome low nutrient conditions. For instance, inactivating mutations in GATOR1, an upstream inhibitor of the Rag GTPases, are frequent in cancer, leading to hyperactive mTORC1 signalling and tumour cells becoming insensitive to amino acid starvation (Bar-Peled et al., 2013). Although, as previously stated, the role of PAT4 in mediating resistance to glutamine-depletion is still highly speculative. Nonetheless, it is interesting to consider the potential clinical implications of this theory if true.

Interestingly, the importance of glutamine metabolism in PCa progression has only recently been highlighted. As PCa advances, alterations in cellular metabolism have frequently been reported in tumours, including increased glutamine metabolism (Fendt et a., 2013). It has recently been shown that PCa cells are addicted to glutamine metabolism under conditions of nutrient stress and during disease progression (Dasgupta et al., 2015), and that inhibition of this pathway can decrease tumour growth and metastatic spread. Hence, if PAT4 is involved in the sensing of glutamine in PCa as it is in colorectal cancer, then blocking PAT4 function may prove an effective clinical strategy to halt PCa progression by depriving PCa tumours of the signals necessary to sustain cell growth. Such a theory therefore warrants further investigation.

Although the discussions above highlight PAT4 as a promising clinical target, additional findings presented in this chapter indicate that inhibiting PAT4 function may affect the type of EVs secreted from PCa cells. Whilst the effects of PAT4 knockdown on EV signalling was not examined directly, both glutamine-depletion and mTORC1 inhibition altered the protein profile of EVs secreted from PCa cells. Hence, moving forwards, it will be important to define the function of these so-called 'switched' EVs in order to fully understand the clinical implications of targeting PAT4 therapeutically.

CHAPTER 5

RESULTS

5. Exploring the regulation of EV signalling by microenvironmental stress in PCa

5.1. Introduction

5.1.1 EV heterogeneity and function in cancer

Normal cells and cancer cells secrete a heterogeneous population of EVs that can differ in their molecular composition, biophysical features, and/or routes of biogenesis. Such a strategy likely enables cells to simultaneously communicate with multiple other cell types and engage a host of different cellular processes to orchestrate a coordinated physiological response. Indeed, in recent years it has been increasingly reported that different EV populations regulate distinct biological events. Such a concept has predominantly been examined in the context of cancer, where distinct EV populations and subpopulations have been implicated in facilitating different processes during tumorigenesis. One study, for example, demonstrated via comparative analysis of EVs derived from melanoma cells, that distinct EV types differed in their pro-coagulant and immunogenic properties (Muhsin-Sharafaldine et al., 2016). Specifically, apoptotic bodies were revealed as more powerful inducers of coagulation than either microvesicles or exosomes, and uniquely able to protect against tumour formation *in vivo*. Likewise, another study showed that exosomes isolated from neuroblastoma cells could stimulate cell proliferation and migration to a greater extent than microvesicles *in vitro* (Keerthikumar et al., 2015). Differences in the functional effects of distinct vesicle subtypes within these EV populations have also been observed. For instance, two different exosome subpopulations isolated from melanoma cells were shown to regulate the expression of a disparate set of genes in recipient endothelial cells (Willms et al., 2016); whereas another study, using the same melanoma cell line, identified two additional exosome subpopulations and revealed a distinct organ biodistribution pattern for each *in vivo* (Zhang et al, 2018). Hence, the way in which different EV subtypes stimulate

disparate functional outcomes may depend on the specific biological pathways EVs engage in recipient cells, and/or the specific recipient cells to which EVs are targeted.

5.1.2 Current issues in the development of EV-based therapeutics

Altogether, the above observations indicate that different EV populations and exosome subtypes are secreted by cells to fulfil distinct biological purposes and may, therefore, help to mediate the pleiotropic effects of cancer. For these reasons, it has become increasingly important for groups within the EV field to characterise the type of vesicles they have isolated in order to provide more definitive conclusions about the functional roles distinct EV populations play in disease progression. As I mentioned earlier (section 1.3.4.3.6), the development of new EV isolation techniques has been instrumental in improving our ability to characterise individual EV populations. In the future, another significant challenge will be to uncover the mechanisms underpinning the regulation of specific EV subtypes. Indeed, by characterising the different EV populations and subpopulations that exist, their functional roles in health and disease, as well as the signalling pathways they are regulated by, we may be better positioned to unlock the true therapeutic potential of EVs.

In Chapter four, I showed that nutrient deprivation as well as pharmacological inhibition of mTORC1, could each promote the secretion of EVs marked by Rab11 from PCa cells. Previous work completed by fellow lab members in HCT116 colorectal cancer cells and *Drosophila* has revealed that these vesicles may represent an alternative type of exosome formed within Rab11-marked compartments in the endocytic pathway. However, I have yet to characterise the identity of the EVs secreted from PCa cells under such conditions. I am also yet to establish whether conditions other than nutrient deprivation, which similarly modulate mTORC1 activity, affect EV signalling in a comparable manner.

5.1.3 Aims and objectives

i. Investigate whether other microenvironmental stressors regulate EV signalling in PCa
ii. Characterise the type of EVs secreted by PCa cells during the exosome switching event
iii. Examine the function of different EV subtypes in PCa progression

5.2 Results

5.2.1 Establishing conditions to isolate EVs from cells treated with anti-androgens

As discussed in Chapter 4, crosstalk between the AR and mTORC1 has previously been shown to play an important role in regulating PCa cell growth. Indeed, one of the ways in which androgens stimulate PCa cell growth is by enhancing the expression of a host of genes that promote mTORC1 activity (e.g. PAT4). It is therefore unsurprising that anti-androgen treatment has been shown to decrease mTORC1 signalling in LNCaP cells. Considering the work presented in the previous chapter revealed that inhibition of mTORC1 activity altered the type of EVs secreted from LNCaP and C4-2B cells, it is possible that inhibition of AR signalling may drive similar changes in EV cargo. To test this theory, I decided to block AR activity in PCa cells and examine the secreted EVs for changes in their protein signature.

Initially, a drug screen was performed to determine the concentration of androgens and anti-androgens required to produce the greatest change in mTORC1 activity between the control and experimental conditions. I reasoned that, if mTORC1 is responsible for regulating the changes observed in EV cargo, then such a strategy may make it easier to detect such differences. In terms of cell lines, the screen was performed in LNCaP and VCaP cells as both lines are androgen-sensitive, a necessary feature to examine the effects of blocking androgen signalling. In addition, given I had previously characterised the effects of inhibiting mTORC1 activity on EV signalling in LNCaP cells, as well as the role of PAT4 in VCaP cells, it seemed appropriate to continue using these cell lines to investigate the effects of anti-androgens on mTORC1 activity and EV signalling.

Considering the ultimate aim of this work was to characterise changes in EV cargo following androgen deprivation, two further parameters I set for this analysis were to perform the screen in conditions that were both androgen-depleted and suitable for EV isolation.

Therefore, cells were grown in their basal media supplemented with 1% ITS as this condition is serum- and androgen-free, and I had previously shown it to be sufficient in maintaining mTORC1 activity as well as LNCaP and VCaP cell growth over a 24-hour period (Figure 5.1).

For the screen, cells were treated with a concentration range of DHT alone (as indicated), or in combination with the anti-androgen bicalutamide (10 μM) for 24 hours. A range of androgen concentrations were examined to determine the condition which produced the greatest reduction in mTORC1 activity following co-treatment with bicalutamide. Previous studies had suggested that the concentration of this anti-androgen must exceed the androgen concentration by at least 1000-fold to sufficiently prevent AR activation, therefore, 10 μM bicalutamide was selected as it represented a concentration which could reportedly block AR signalling in all of the DHT concentrations examined in this analysis. Unfortunately, I could not confirm this response by assessing AR activity directly due to difficulties encountered with the PSA antibody. I did, however, find that the concentration of bicalutamide eventually selected could effectively inhibit PCa cell growth (data not shown), suggesting that the drug was successfully blocking AR signalling.

Consistent with the published literature, the addition of DHT increased mTORC1 activity in a concentration-dependent manner in both LNCaP and VCaP cells, as evidenced by the increase in pS6 in both cell lines, and hyperphosphorylated 4E-BP1 in VCaP cells (Figure 5.1A). However, although co-treatment with bicalutamide inhibited the phosphorylation of S6 in LNCaP and VCaP cells when the cells were cultured in high concentrations of DHT (i.e. 1 nM or above), no decrease in hyperphosphorylated 4E-BP1 was observed in any condition following bicalutamide treatment in either cell line after 24 hours. Therefore, I decided to modify the culture conditions in an attempt to replicate the mTORC1 signalling profile that had previously produced the greatest change in EV signalling (i.e. decreased

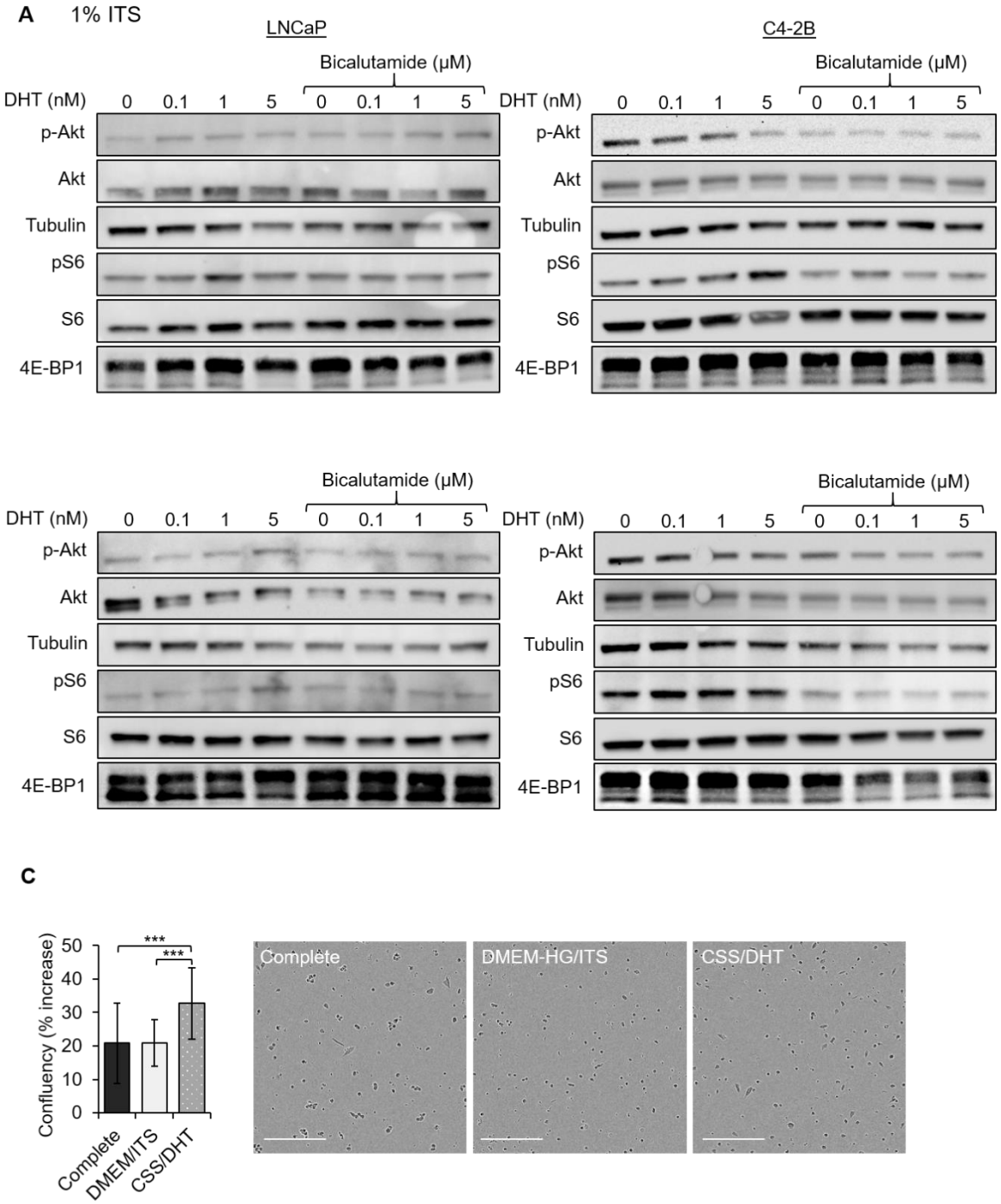


Figure 5.1. Establishing media conditions to isolate EVs from VCaP and LNCaP cells following anti-androgen treatment. Western blot showing the dose-response of mTORC1 signalling in each cell line to DHT stimulation alone, or in combination with bicalutamide (10 μM), when the media was supplemented with ITS **(A)** or CSS **(B)** for 24 hours. **(C)** VCaP cells were cultured in the indicated media for 24 hours before the effects on cell growth (left panel) and morphology (right panel) were assessed. To monitor cell growth, phase-contrast images were collected every two hours using the IncuCyte ZOOM^{TM}. Cell growth is represented as the fold change in percentage confluency of between 0 and 24 hours. Results are mean ±S.D of three experiments (n = 8 per condition). ***P < 0.001 vs. control.

pS6 and hyperphosphorylated 4E-BP1). I reasoned that the high concentration of ITS present in the culture medium may have led to saturating levels of growth factors, and that any changes in mTORC1 signalling caused by inhibition of AR activity could be difficult to detect as a result.

To combat this issue, I decided to supplement the media with 1% pre-spun CSS instead and assess whether reduced levels of growth factors could enable more subtle changes in mTORC1 signalling to be detected. Additionally, I chose these conditions as they satisfied the parameters I had previously set for this analysis; that is, the serum had been charcoal-stripped and ultracentrifuged to deplete the medium of both steroid hormones and contaminating EVs, respectively.

Overall, culturing LNCaP cells in the medium supplemented with CSS produced similar results to those observed with ITS. For instance, treatment with androgens increased mTORC1 activity in a concentration-dependent manner, whereas the addition of bicalutamide inhibited the phosphorylation of S6 more than 4E-BP1 (Figure 5.1B right panel). Conversely, in VCaP cells, although the response was similar to that observed with ITS, a greater decrease in the phosphorylation of 4E-BP1 was seen following bicalutamide treatment (Figure 5.1B left panel). Presumably, VCaP cells are more sensitive to low serum, hormone-depleted culture conditions than LNCaP cells. Indeed, in VCaP cells, basal mTORC1 activity could only be maintained over the 24 hour incubation period following the addition of high concentrations of androgens (5 nM DHT), as shown by the increased proportion of hyperphosphorylated 4E-BP1 in this condition compared to the others. On the other hand, in LNCaP cells, the majority of 4E-BP1 appeared to be hyperphosphorylated even when the cells were treated with the lowest concentration of androgens (0.1 nM); a result, perhaps, of the hyperactive PI3K/Akt/mTORC1 signalling in these cells caused by loss of PTEN. Therefore, it seemed that androgens were more important for sustaining mTORC1 signalling in VCaP cells in these culture conditions - a finding which could

potentially explain why inhibition of AR signalling decreased mTORC1 activity to a greater extent in VCaP than LNCaP cells.

I also noted that treatment with androgens differentially affected mTORC2 activity in both cell lines. For instance, increasing concentrations of DHT promoted Akt phosphorylation in VCaP cells yet inhibited this action in LNCaP cells; whereas co-treatment with bicalutamide decreased overall p-Akt levels in both cell lines compared to control (Figures 5.1A and 5.1B). Reviewing my work presented in Chapter 4; treatment of LNCaP or C4-2B cells with Torin1 (the condition which produced the greatest change in EV signalling), also inhibited mTORC2 activity, consistent with the effects of bicalutamide shown here for VCaP cells. Therefore, based upon these observations, I decided to continue the EV analysis in the VCaP cell line as treatment with anti-androgens appeared to produce similar changes in mTOR signalling that had previously been shown to correlate with the exosome switch phenotype in other PCa cell lines.

5.2.2 Validating the EV isolation protocol

As I had not yet isolated EVs from cells that had been cultured in a medium containing serum or from the VCaP cell line, I believed it was important to first demonstrate that the culture conditions and cells that I had chosen were both suitable to use for this analysis. Initially, I began by examining the growth of VCaP cells cultured in the newly-developed EV isolation medium (DMEM, 1% pre-spun CSS, 5 nM DHT), to ensure that they were healthy and behaving similarly to those grown in standard culture conditions. Indeed, I had already shown that mTORC1 signalling remained active in VCaP cells cultured in the low serum, high androgen medium for 24 hours, indicating that the cells were not stressed during the period of EV collection (Figure 5.1B, left panel). In terms of growth, VCaP cells cultured in this medium were approximately 50% more confluent than those grown in standard media (i.e. plus 10% FBS) after 24 hours, suggesting that the level of androgens rather than the level of nutrients normally limits VCaP cell growth (Figure 5.1C left panel). Indeed, the

confluency of VCaP cells cultured without androgens, but with high levels of growth factors (i.e. DMEM plus 1% ITS), was approximately 30% less than that of VCaP cells cultured in low-serum, high androgen conditions; further highlighting the importance of androgens in driving VCaP cell growth. Moreover, morphologically, cells grown in the absence of androgens appeared more rounded in shape compared to those cultured in media containing FBS or DHT (Figure 5.1C, right panel), again suggesting that androgens are necessary to maintain a healthy VCaP cell population. Therefore, I concluded that the DMEM/CSS/DHT culture medium was suitable to use to isolate EVs from VCaP cells after 24 hours.

Next, having decided upon an appropriate isolation medium, I wanted to assess whether EVs could be purified from VCaP cells cultured in these conditions. As I had had previous success using SEC to isolate EVs from LNCaP and C4-2B cells, I decided to use this method again to isolate vesicles from VCaP cells. Using this technique, EVs secreted from VCaP cells were isolated from conditioned cell culture medium after 24 hours and characterised to determine sample purity (Figure 5.2). In terms of protein cargo, immunoblotting revealed that the canonical exosome markers Alix, syntenin-1, CD81 and CD63 were all highly enriched in EVs purified from the conditioned medium compared to cell lysate (Figure 5.2B, left panel). Similarly, Tsg101 and Flotillin-2, two other positive exosome markers, were also observed in these EVs, whereas Calnexin, LAMP-1 and EEA1 were absent, signifying that the sample preparation was pure. Consistently, NTA revealed that most of the vesicles isolated via SEC measured between 110-120 nm, with very few exceeding 200 nm (Figure 5.2B, right panel), indicating that the samples were devoid of large, contaminating particles (e.g. microvesicles, cell debris). This finding was further

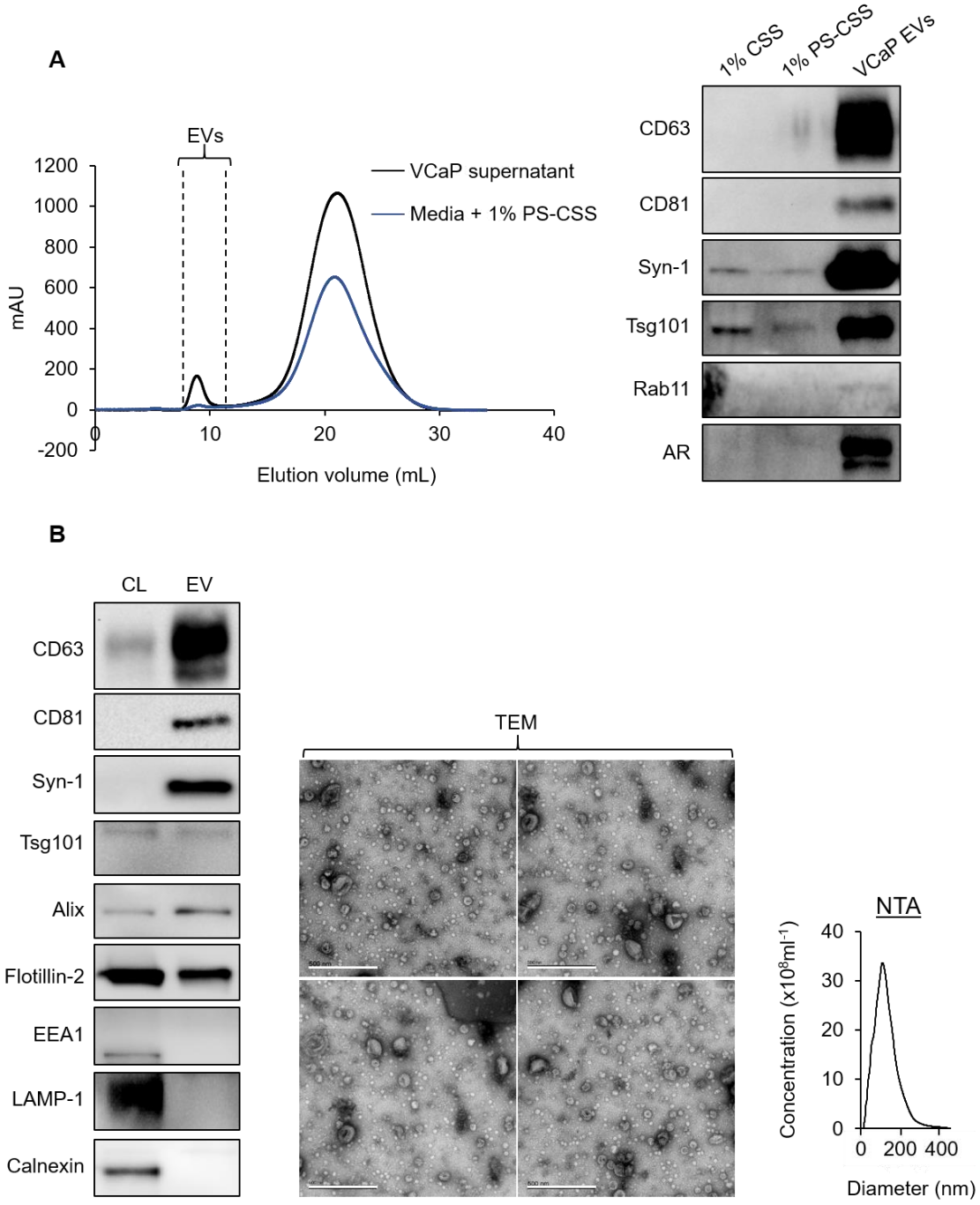


Figure 5.2. Characterisation of extracellular vesicles (EVs) isolated from VCaP cells and culture medium. **(A)** VCaP conditioned medium and medium containing CSS (1%), both pre-spun and unprocessed, were separated via SEC (**left** panel) and analysed for several exosome markers via western blot (**right** panel). **(B)** EVs were isolated from VCaP cells cultured in medium supplemented with 1% pre-spun CSS after 24 hours using size exclusion chromatography (SEC). EV protein and cell lysate (CL; 3.5 µg) were analysed via western blotting for several exosome-specific and -non-specific markers (**left** panel). EV morphology and size were examined via transmission electron microscopy (TEM; **middle** panel) and nanoparticle tracking analysis (NTA; **right** panel), respectively. (Scale bar = 500 nm).

supported by TEM analysis (in collaboration with Dr Errin Johnson) which demonstrated that the preparations contained a heterogeneous mixture of intact vesicles, many of which displayed the classical cup-shape morphology and measured approximately 30 to 120 nm in diameter (Figure 5.2B, middle panel). Overall, these findings indicated that SEC was a reliable method to isolate a population of EVs from VCaP cells that likely included exosomes, and could therefore be used for further analysis.

So far, the conditions I had established to block mTORC1 signalling in VCaP cells following anti-androgen treatment also appeared suitable for the isolation of EVs. However, given that the isolation medium I had developed contained serum, albeit low levels that had been ultracentrifuged, a question remained as to whether any serum-derived vesicles remained. Considering that I ultimately wanted to assess whether EV signalling was altered in VCaP cells following anti-androgen treatment, it seemed important to first establish the origin (i.e. serum or cell) of the EV cargo I was interested in. Indeed, if the protein signal from the serum was particularly strong, then subtle changes in EV cargo could be difficult to detect. To test this, I decided to compare the abundance of protein markers present in non-conditioned isolation medium to those present in the medium used to culture VCaP cells for EV isolation. In this way, the relative contributions of serum- and cell-derived EV proteins could be determined. For comparison, the same volume of DMEM medium as used to culture VCaP cells - also supplemented with 1% pre-spun CSS and 5 nM DHT - was processed in an identical manner to the conditioned medium (i.e. 500 x g then 2000 x g centrifugation; 0.22 µm filtration; TFF; ultrafiltration) before EVs were isolated via SEC (Figure 5.2A).

The chromatograms produced from this analysis revealed that the UV profiles differed greatly between the samples obtained from the conditioned and non-conditioned media. Importantly, a large peak corresponding to fractions 2-5 (i.e. the fractions reportedly enriched for exosomes) was present in the sample from the conditioned medium yet

considerably diminished in the sample from the non-conditioned medium, suggesting that the majority of EVs found in the final preparation were secreted from VCaP cells (Figure 5.2A, left panel). Consistently, all of the EV markers examined were either heavily enriched for, or detectable solely, in those vesicles isolated from the conditioned medium, again indicating that serum-derived EVs contribute very little to the overall sample (Figure 5.2A, right panel). Therefore, given it seemed unlikely that the serum present in the isolation medium would hinder the EV analysis, I decided to examine whether inhibition of AR signalling could alter the protein profile of EVs secreted from VCaP cells using these conditions.

5.2.3 Androgen-deprivation induces EV switching in VCaP cells

For EV isolation, VCaP cells were cultured in DMEM medium supplemented with 1% CSS plus 5 nM DHT and treated with either vehicle (i.e. DMSO) for the control condition or bicalutamide (10 μM) for the androgen-depleted conditions for 24 hours. To confirm that the cells were responding to anti-androgen treatment in the same manner as previously characterised, cell lysates were analysed by western blotting and the level of mTORC1 activity was assessed (Figure 5.3). Consistent with the dose-response data, compared to VCaP cells cultured in media supplemented with DHT alone, cells maintained in androgen-depleted conditions displayed reduced levels of both S6 and 4E-BP1 phosphorylation (Figure 5.3A, right panel), confirming the efficacy of the drug treatment. In addition, no significant difference in the expression of any of the EV protein markers was observed in the cell lysate following bicalutamide treatment, indicating that any changes in EV cargo would likely reflect differences in vesicle regulation, not total protein.

As was established for the analysis of EVs isolated from LNCaP and C4-2B cells, EVs derived from VCaP cells were similarly examined via western blotting by normalising the volume of sample loaded to the total protein content of the cells. The level of Tsg101 in EVs appeared consistent in both control and androgen-deprived conditions, therefore this

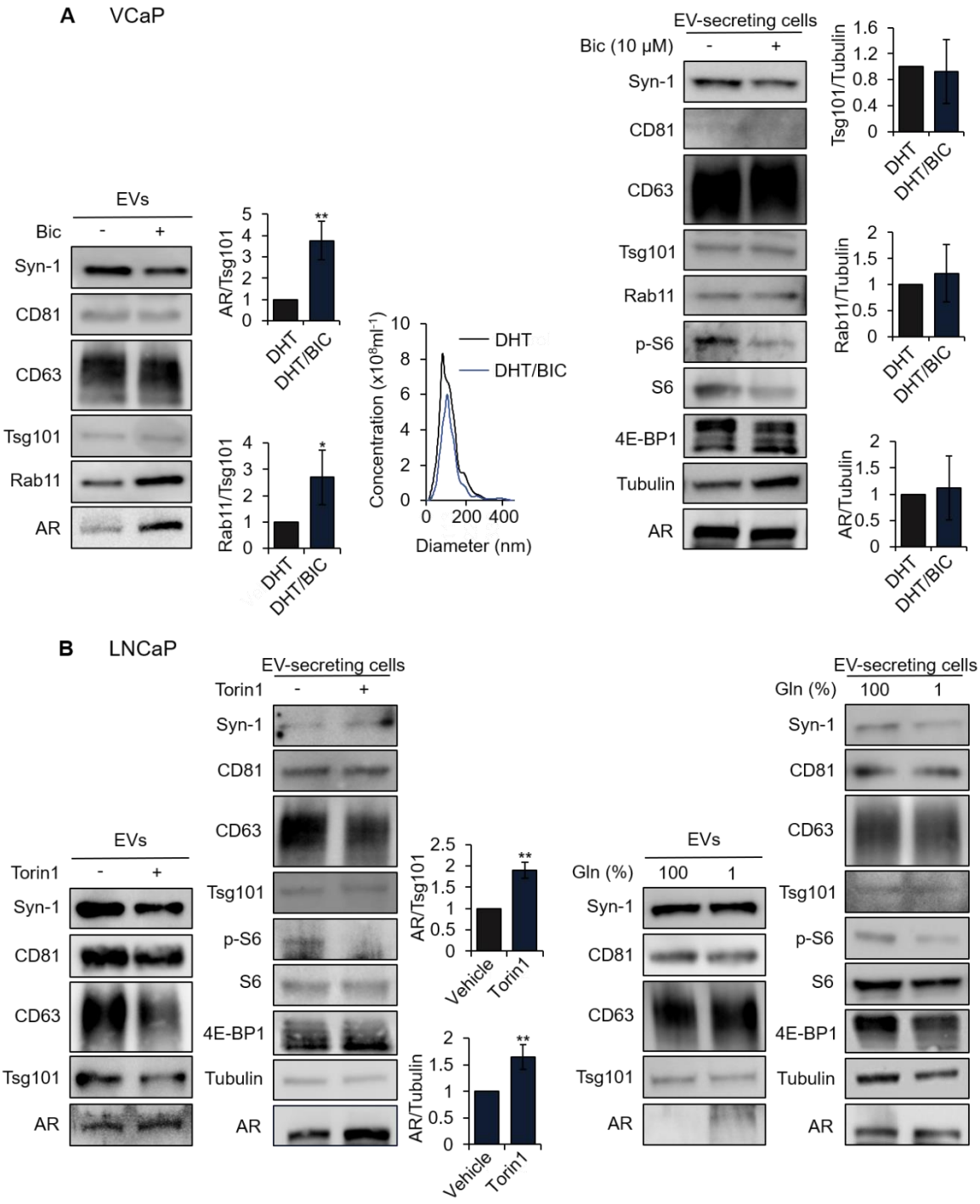


Figure 5.3. Conditions of mTORC1-mediated microenvironmental stress enhance the secretion of AR from PCa cells. **(A)** VCaP cells were treated with DHT alone (0.1 nM) or with bicalutamide (10 μM) for 24 hours before the effects on mTORC1 activity and the expression of several canonical and non-canonical (i.e. AR) exosome proteins were examined in the cell lysate via western blotting (**right** panel). EVs were isolated from these cells after the same period and characterised for changes in the level of these proteins via western blotting (**left** panel) and size via NTA (**middle** panels). **(B)** AR expression was also examined in LNCaP cells and EVs following Torin1 treatment (**left** panels) and glutamine depletion (**right** panels). Results are mean ±S.D of three independent experiments (n = 1 per condition). *P < 0.05 and **P < 0.01 vs. control.

marker was chosen to serve as the loading control for this analysis. Additional cargo unaffected by bicalutamide treatment included syntenin-1, CD81 and CD63; however, intriguingly, Rab11 was significantly increased following anti-androgen treatment (Figure 5.3A, right panel). Analysis of the EV preparations using NTA revealed minor differences in vesicle size and number, suggesting that cells cultured in androgen-deprived conditions secrete a similar amount of EVs to control cells, enriched for a different set of cargoes (Figure 5.3A, middle panel). Intriguingly, these changes in EV cargo paralleled those observed in LNCaP cells following glutamine depletion, suggesting that androgen-deprivation may represent another condition that can regulate the exosome switching event in PCa cells. These findings further indicate that this regulatory pathway may exist in multiple PCa cell types and be influenced by a multitude of extracellular stimuli. Although the importance of mTORC1 in mediating the effects of glutamine or anti-androgens on EV secretion remains unknown, it is interesting that, in VCaP cells, bicalutamide appeared to affect mTORC1 activity in a similar manner to glutamine depletion in LNCaP cells, and that both treatments produced similar changes in EV cargo (i.e. increased Rab11, unchanged CD63). This finding further highlights the association between changes in the mTORC1 signalling profile and the type of EVs secreted from these cell lines, and again, points to a possible role for this signalling pathway in underpinning these events.

5.2.4 mTORC1 inhibition induces AR secretion in LNCaP cells

In addition to examining the EV markers previously established as representative of the exosome switch phenotype in HCT116 cells, I decided to screen for additional cargo important in PCa to understand how changes in EV signalling may affect disease progression. Of particular interest to me was the AR due to its pivotal role in PCa progression. Moreover, at the time of analysis, only one other study had reported this protein in vesicles, therefore the AR appeared to be a novel EV marker with great clinical potential. Considering I had already shown that AR signalling was involved in regulating EV cargo, I wondered whether this pathway could also alter the secretion of AR-positive EVs.

After confirming that I could detect the AR in EVs isolated from VCaP cells using SEC, I went on to investigate whether the level of AR changed following anti-androgen treatment (Figure 5.3). Interestingly, I found that the level of extracellular AR increased almost four-fold following bicalutamide treatment and that this response did not reflect a change in AR protein expression, suggesting a specific change in the regulation of these specific EVs instead (Figure 5.3A). Overall, these findings led me to question whether increased secretion of the AR may be a feature of the exosome switch phenotype in PCa cells. To test this, I decided to re-examine the cargo of EVs derived from LNCaP cells cultured in conditions which had previously produced the switch for evidence of similar changes in the AR.

As discussed in the previous chapter, inhibition of mTORC1 signalling by Torin1 produced the greatest change in EV cargo that I had observed. Therefore, I reasoned that any changes in the secretion of AR may be easier to detect in EVs isolated from PCa cells cultured in these conditions. In LNCaP cells, Torin1 treatment significantly increased in the secretion of AR-positive EVs, although to a lesser extent than that which was observed in VCaP cells treated with bicalutamide (Figure 5.3A, left panels). Also, unlike the response observed in VCaP cells, Torin1 treatment enhanced the expression of AR in the LNCaP cell lysate. It was therefore unclear whether the increased AR in EVs was a non-specific effect of there being more total AR protein in the cell. However, analysis of EVs derived from glutamine-depleted LNCaP cells also revealed increased levels of AR, whilst total AR protein remained unchanged, suggesting a level of selectivity regarding the trafficking of this cargo into EVs in these cells (Figure 5.3B, right panels). Nonetheless, since the AR has not yet been established as a bona fide EV cargo protein, I decided to assess whether the AR identified in the SEC preparations was associated with vesicles and, therefore, a component of the switched EV signature in PCa cells.

5.2.5 AR released by LNCaP cells is associated with EVs

To assess whether the AR I had identified in the conditioned PCa cell culture medium was EV-derived, I decided to examine the behaviour of this protein compared to other well-characterised EV cargoes. Overall, I wanted to confirm that the alterations in AR secretion I had discovered reflected specific changes in EV regulation and not other cellular responses (e.g. increased apoptosis). Initially, I believed it was important to first establish whether extracellular AR was membrane-bound, as this appeared to me the most fundamental characteristic of a genuine EV cargo. Encouragingly, I found that the AR identified in the LNCaP supernatant was resistant to trypsin digestion in the absence but not in the presence of detergent, confirming that extracellular AR was located within a membrane-bound particle (Figure 5.4C). Supporting this finding, the same response was also observed for syntenin-1, a well-characterised intra-exosomal cargo protein. Following this result and given the fact that the exosome switch phenotype in HCT116 cells involves changes in *exosomal* cargo, I next wanted to establish the identity of the AR-positive EVs.

As different types of EVs can be distinguished by properties such as size, shape and density, I decided to assess whether the biophysical features of AR-positive vesicles resembled any previously-characterised EV types. Initially, I attempted to compare the density of extracellular AR to other well-established EV markers using sucrose density gradient UC. Unfortunately, using this technique, I could only detect the AR in the input sample and not in any of the fractions collected following centrifugation in the sucrose gradient (data not shown). Given that the basal level of AR in EVs isolated from this cell line using UC was low (Figure 5.4D), I reasoned that subjecting these preparations to further separation techniques may have yielded too few AR-positive vesicles for detection via western blot. Instead, I decided to adopt a different strategy and separate vesicles based upon their size, then interrogate the resultant SEC elution profile for different EV markers.

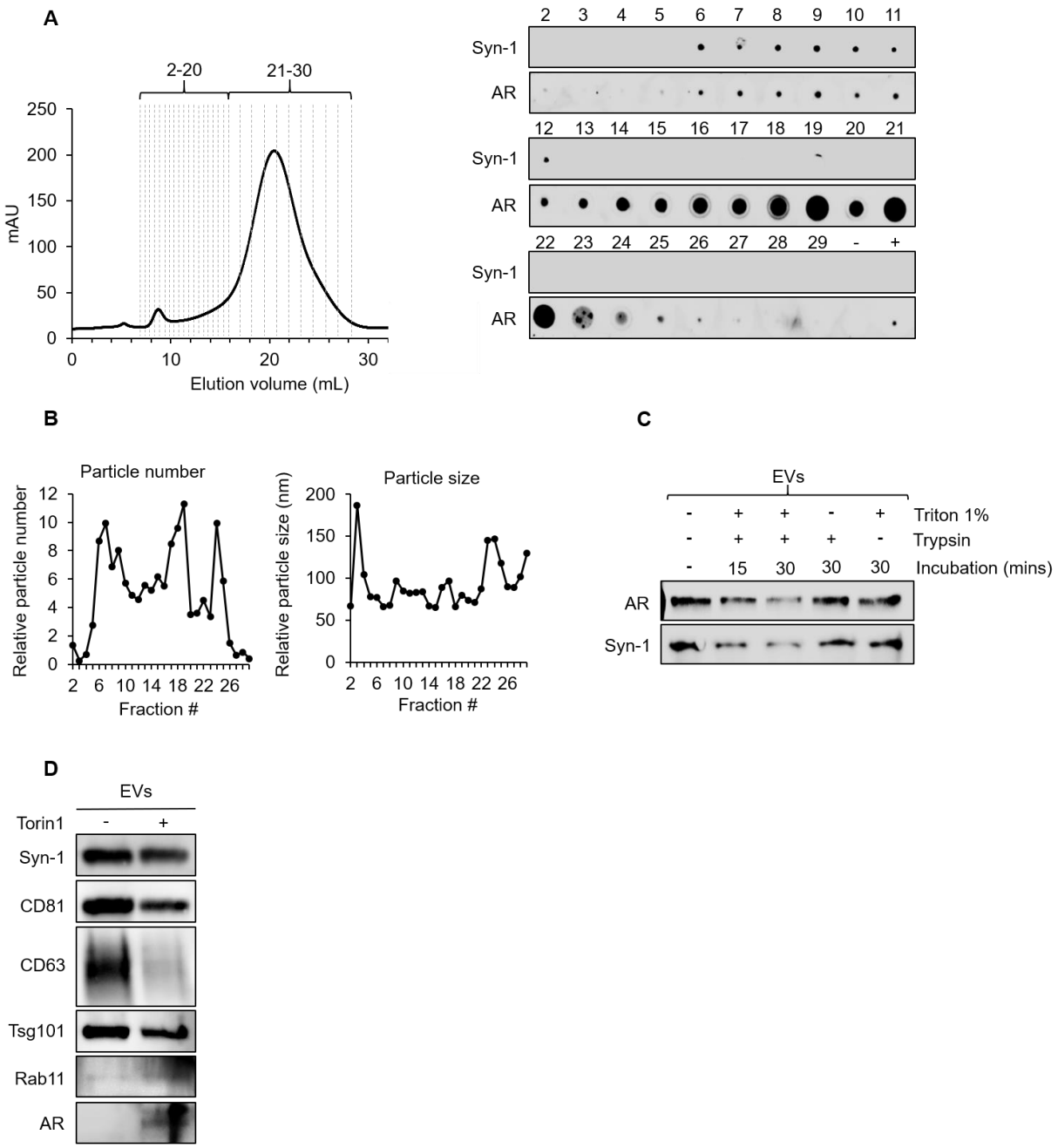


Figure 5.4. The AR in LNCaP conditioned medium is membrane-bound and marks a heterogeneous population of EVs. **(A)** The supernatant from LNCaP cells cultured in standard conditions was collected after 24 hours and separated via SEC (**left** panel) before each fraction was analysed via dot blot for AR and syntenin-1 (**right** panel). Negative control = PBS; positive control = unprocessed supernatant. **(B)** Each fraction was analysed via NTA for average particle size and number. **(C)** EVs were isolated from LNCaP cells cultured in standard conditions after 24 hours via UF, then treated as indicated before AR and syntenin-1 levels were analysed via western blot. **(D)** LNCaP cells were treated with vehicle (DMSO) or Torin1 (120 nM) for 24 hours before EVs were isolated via UC and their exosomal cargo analysed via western blot.

Conditioned LNCaP cell culture media was fractionated via SEC and the protein composition of each individual fraction was analysed via dot blot with the help of DPhil student Eduard Willms (Wood lab; Figure 5.4A). Interestingly, although I found that the AR co-eluted with the well-established exosome marker syntenin-1 in the initial UV peak (i.e. fractions 6-12), I discovered that it was predominantly enriched for in the fractions found between this peak and the second (i.e. fractions 13-22), signifying that extracellular AR may be associated with a heterogeneous population of EVs, a proportion of which may include exosomes. Indeed, in terms of size, particles eluted within fractions 13-22 could still potentially fall within the reported exosomal size range (i.e. 30-120 nm). Given that the SEC column is packed with a resin capable of resolving particles between 6-40 nm (pore size = 84 nm), the first peak on the chromatogram likely corresponds to the elution of larger particles present within the supernatant that exceed 84 nm (e.g. EVs), whereas the second UV peak presumably reflects the emergence of molecules smaller than 40 nm (e.g. free protein). Therefore, particles that measure within this size range (i.e. 40-84 nm) may elute somewhere between these two peaks (Figure 5.4A, left panel). However, when each fraction was analysed via NTA, no such pattern was observed (Figure 5.4B). In fact, in not one fraction did the average particle size measure less than 50 nm, perhaps reflecting the limitations of this technique and/or protein aggregation within the samples. Nonetheless, whilst these findings failed to reveal the specific type of vesicles marked by the AR, they did raise the interesting possibility that such an EV panel may include a range of both classical and non-classical exosomes.

By this stage, I had successfully detected the AR in EVs isolated using both SEC and UF. However, given that UC remained the gold-standard for exosome isolation, I decided to repeat my previous analysis of the LNCaP cell culture medium using this method. As before, LNCaP cells were cultured in RPMI medium supplemented with 1% ITS and treated with either vehicle (i.e. DMSO) or Torin1 (120 nM) for 24 hours. The conditioned medium was initially processed in a similar manner to SEC (i.e. 500 x g then 2000 x g centrifugation;

0.22 µm filtration; TFF) before being subjected to high-speed UC for two hours. Following a wash step, the pellet was resuspended in PBS and analysed via western blot. Overall, the AR was undetectable in EVs isolated from control LNCaP cells whereas syntenin-1 was clearly visible, in contrast to the SEC data which indicated that AR was the more prominent marker in the LNCaP supernatant. Hence, these observations further suggested to me that AR may mark a different EV population to syntenin-1 - one presumably not enriched for during UC. However, following Torin1 treatment, AR levels were clearly enhanced within EVs, suggesting that either (i) the level of AR within the EV type previously pelleted had increased, (ii) the secretion of a different AR-positive EV population was induced by the treatment, or (iii) both. Interestingly, a similar pattern was observed for Rab11, raising similar questions for this marker.

5.2.6 AR released by LNCaP cells is functional

The findings so far indicated that extracellular AR was membrane-bound and associated with a heterogenous population of EVs. Next, I wanted to test whether the AR within these vesicles was taken up by, and able to function within, recipient cells. Given a key process that is regulated by AR signalling is cell proliferation, I decided to examine the effects of these EVs on PCa cell growth. Experimentally, LNCaP cells were treated with EVs isolated from control or mTORC1-inhibited LNCaP cells, and proliferation was monitored using the IncuCyte ZOOM™ over 96 hours (Figure 5.5A). Interestingly, in androgen-depleted medium, the addition of EVs isolated from cells cultured in either condition decreased LNCaP cell proliferation at every time point compared to cells treated with vehicle (Figure 5.5A, left panels). However, when DHT (0.1 nM) was added to the medium, the opposite effect was observed; ‘switched’ EVs enhanced LNCaP cell growth, perhaps suggesting that only in the presence of ligand could EV-derived AR function effectively (Figure 5.5A, right panels). Indeed, treatment with EVs derived from control cells significantly increased LNCaP cell proliferation at all time points after 48 hours, as did treatment with EVs isolated from mTORC1-inibited cells after 24 hours, although to a

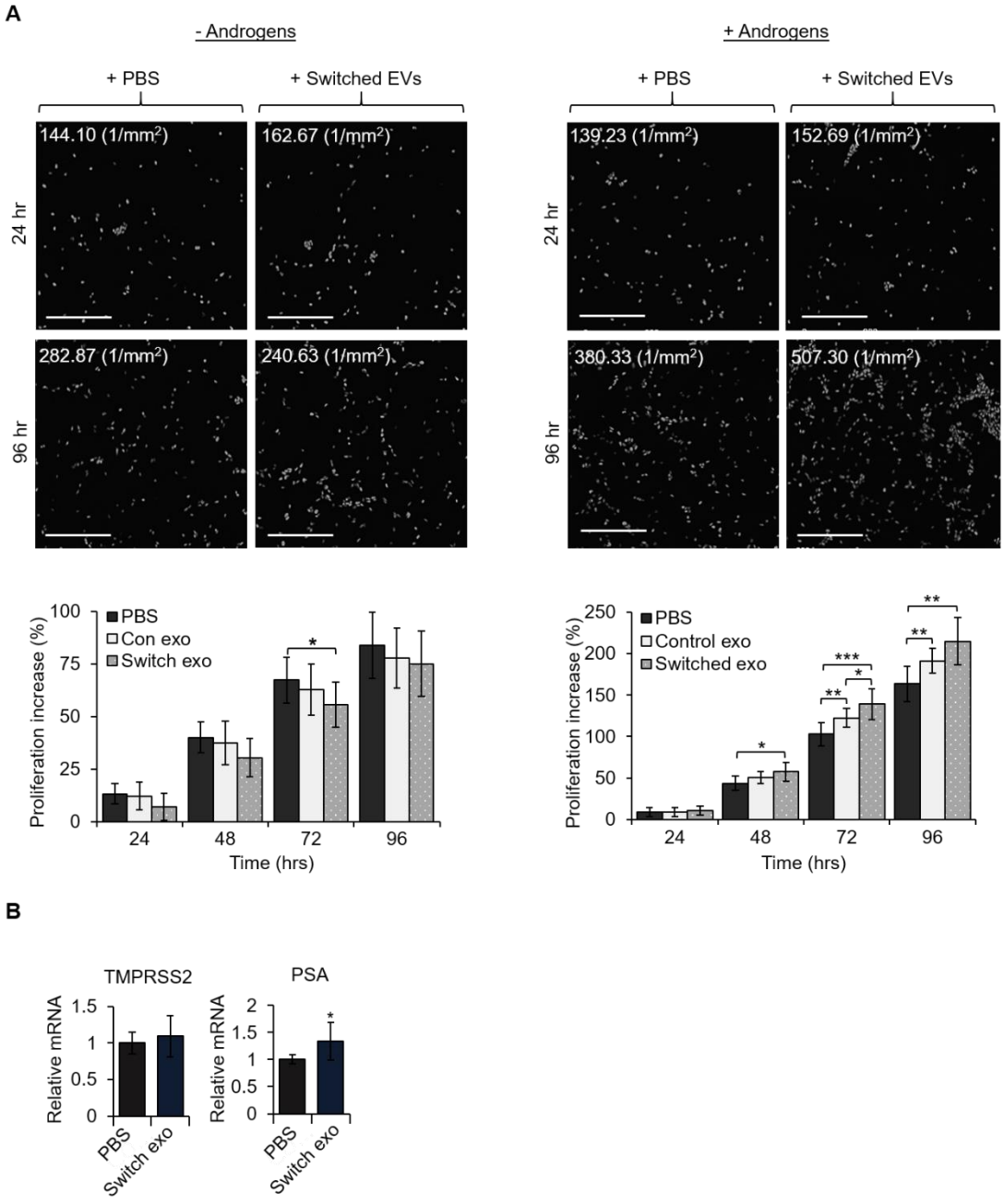


Figure 5.5. EVs derived from mTORC1-inhibited LNCaP cells alters PCa cell growth. **(A)** NucLight-LNCaP cells were cultured in medium supplemented with 1% CSS alone (**left** panel) or with DHT (0.1 nM; **right** panel). Cells were treated with vehicle (PBS) or with EVs isolated via UF from LNCaP cells treated with the mTOR inhibitor AZD8055D (1 µM) for 24 hours. Fluorescent images of the cells were collected every two hours for a total of 96 hours using the IncuCyte ZOOM™ and the number of objects quantified. Cell proliferation is represented as the fold change in red objects (1/mm^2) between 0 and 96 hrs (**lower** panels). Representative images of cells treated with either PBS or EVs at 0 and 96 hours are shown. Results are mean ±S.D of one experiment (n = 8 per condition). Scale bar = 300 µm. **(B)** Expression of AR-regulated genes TMPRSS2 and PSA were examined in LNCaP cells treated as in (A; right panel) after 96 hours via qRT-PCR. Results are mean ±S.D of three experiments (n = 1 per condition). ***P* < 0.01 or ****P* < 0.001 vs. control.

greater extent. Hence, one possible conclusion could be that, as both types of EVs express the AR, cell proliferation may be driven by the addition of either but is more strongly enhanced by treatment with EVs containing higher levels of AR (i.e. those from mTORC1-inhibited cells).

Indeed, further analysis of AR-regulated gene targets revealed a significant increase in the expression of PSA in LNCaP cells treated with EVs derived from mTORC1-inhibited cells versus control cells (Figure 5.5B). However, this difference was relatively subtle, and no change was observed in the level of another AR target gene TMPRSS2. It is possible that the time after which changes in gene expression were assessed following treatment with EVs was too long (i.e. 72 hours), and the effects were diminished as a result. Nonetheless, altogether these findings suggest that EV-derived AR may be transferred to recipient cells and function as a transcription factor to regulate cell proliferation.

5.2.7 Inhibition of mTORC1 or AR signalling affects a heterogenous EV population

Having shown that the AR may mark several EV subpopulations, and play a role in regulating cell proliferation, I reasoned that it was important to characterise the molecular composition of these different subpopulations and identify those affected by changes in mTORC1 activity and AR signalling.

Initially, I decided to characterise the molecular composition of the AR-positive EV subtypes I had already identified, by re-examining the samples separated via SEC and probing for additional markers. Previously, I had used the dot blot method to examine the elution profile of AR and syntenin-1; however, as this technique does not provide any further biophysical information regarding the protein of interest (e.g. molecular weight), it is limited to the use of very clean antibodies that exhibit low non-specific binding activity. Due to these restrictions, I reasoned that another more desirable approach seemed to pool several of these fractions together for analysis via western blot. However,

given the low protein concentration of each sample and the detection threshold of the western blot technique, it was not possible to examine each fraction individually. Instead, after reviewing the chromatogram and dot blot data, I decided to collect a series of fractions corresponding to the first peak, the second peak, and the area in between, as these fractions appeared the most distinct. The lowest number of fractions pooled together was three as, in my hands, this represented the minimum protein concentration required for detection via western blot. Using this strategy, the conditioned supernatant from LNCaP cells cultured in control conditions (DMSO) or with Torin1, and from VCaP cells treated with vehicle (i.e. DMSO) or bicalutamide, was analysed (Figure 5.6A and 5.6B).

Overall, EVs derived from the LNCaP and VCaP conditioned cell culture media displayed similar elution profiles. Encouragingly, all of the EV markers were detected in the fractions eluted before the second UV peak, where very small particles, such as free proteins, reportedly emerge (Figure 5.6A and 5.6B, lower panels). Indeed, when the protein content was analysed via BCA and compared to particle concentration in this peak, the protein to particle number ratio was ~34 fold greater than that measured in the preceding fractions, suggesting soluble proteins present in the conditioned medium are enriched within the second UV peak. Consistent with this, the well-characterised exosome markers CD63 and CD81 appeared most enriched within the fractions corresponding to the initial UV peak, concordant with literature reports that exosomes are eluted within these first fractions.

Nonetheless, both of these proteins were still highly abundant in fractions 5-7, indicating that classical EVs, including exosomes, may also be eluted after the initial UV peak. Indeed, two other exosome markers, syntenin-1 and Tsg101, were predominantly enriched for within fractions 2-7, again potentially signifying the presence of exosomal vesicles in these fractions. Conversely, these two markers, as well as the tetraspanins,

were only faintly detectable in the last three fractions eluted before the second UV peak, suggesting very few exosomes may be found in this region. Interestingly however, it was within these three fractions that the AR was most highly enriched. Indeed, whilst the AR was also detectable in fractions 2-7, alongside the classical EV markers, the signal was far weaker than that observed in the later fractions, consistent with the dot blot data. Additionally, the elution profile for Rab11 appeared very similar to that of the AR, in both control and treated conditions, suggesting that these two proteins may mark the same EV subpopulation(s). Moreover, whilst a proportion of the EVs marked by the AR or Rab11 exhibited similar biophysical properties (i.e. the migration speed through a SEC column) to the classical exosomal markers, the majority were enriched in these later, seemingly exosome-free fractions. However, the fact that Rab11, a predominantly endocytic protein, was also enriched within these fractions raises the intriguing possibility that AR- and Rab11-positive EVs may represent a unique, previously undefined, exosome population. Indeed, such a theory is consistent with that which has been proposed by our lab to explain the presence of Rab11-marked ILVs in MVEs within HCT116 cells and *Drosophila*.

In terms of changes in EV cargo following androgen deprivation or mTORC1 inhibition, the exosome switch phenotype appeared consistent within all fractions. That is, increased AR and Rab11 and, in the case of Torin1 treatment, decreased CD63, were observed in each of the three pooled fractions in both experimental conditions. Hence, these findings indicate that inhibition of the mTORC1 and AR signalling pathways likely affects the regulation of a heterogenous EV population, which might include both classical and non-classical exosomes.

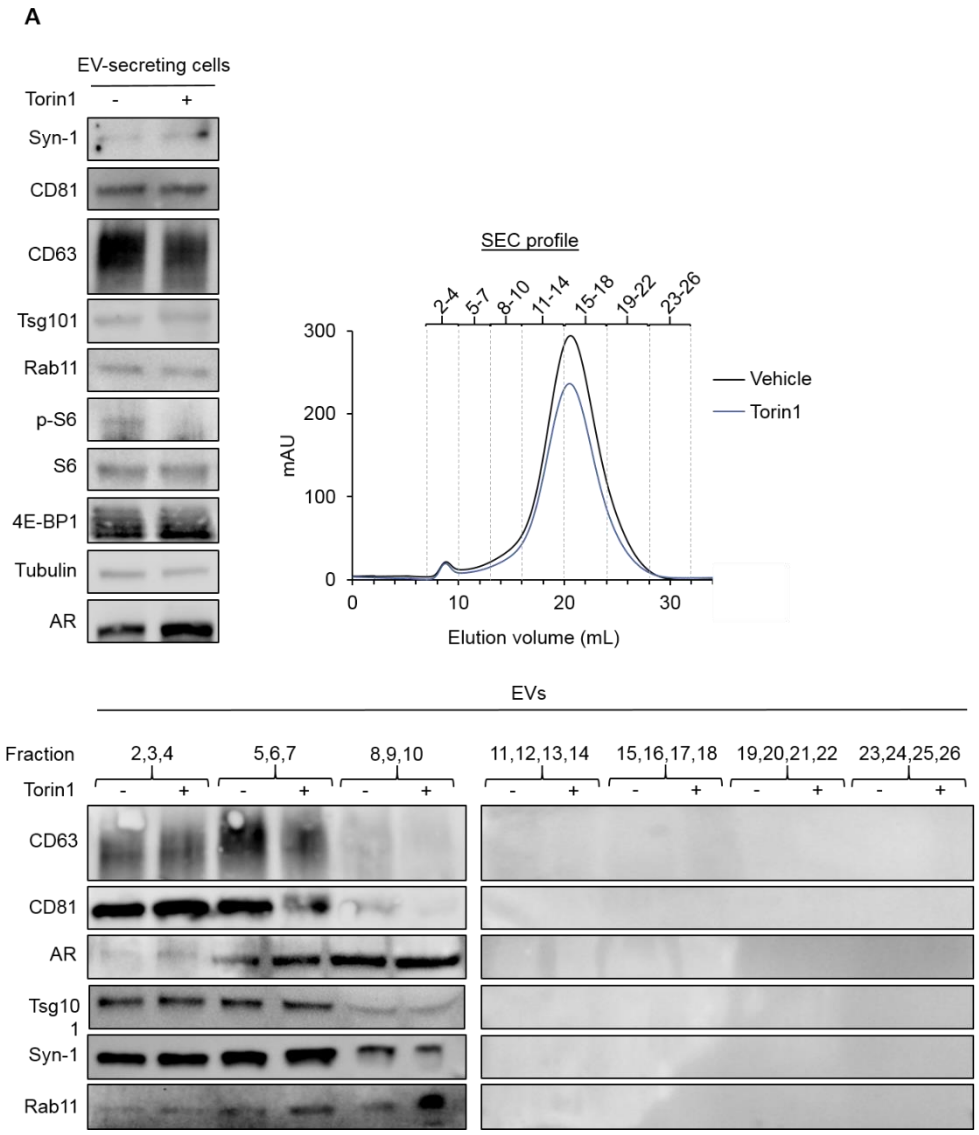


Figure 5.6. PCa cells secrete a heterogeneous population of EVs. **(A)** LNCaP cells were treated with vehicle (DMSO) or with Torin1 (120 nM) for 24 hours before the effects on mTORC1 activity and the expression of several canonical and non-canonical exosome markers were examined in the cell lysate via western blotting (**upper left** panel). The conditioned supernatant from these cells was separated via SEC after the same period and pooled into fractions as indicated (**upper right** panel). These fractions were analysed via western blotting for the same proteins as in the cell lysate (**lower** panel).

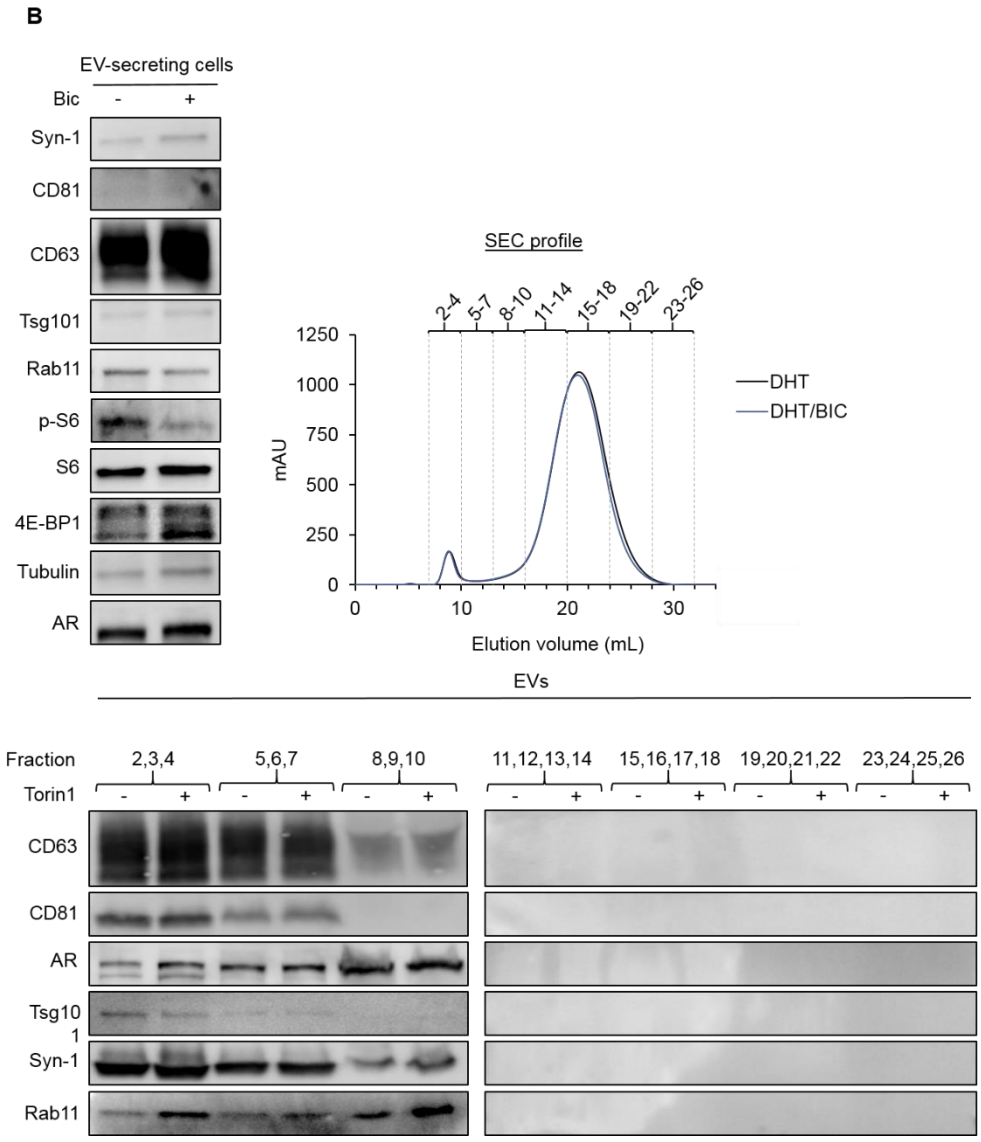


Figure 5.6. (Continued). (B) VCaP cells were treated with DHT (0.1 nM) alone or in combination with bicalutamide (10 µM) for 24 hours before the effects on mTORC1 activity and the expression of several canonical and non-canonical exosome markers were examined in the cell lysate via western blotting (**upper left** panel). The conditioned supernatant from these cells was separated via SEC after the same period and pooled into fractions as indicated (**upper right** panel). These fractions were analysed via western blotting for the same proteins as in the cell lysate (**lower** panel).

5.3. Discussion

In the previous chapter, I showed that inhibition of mTORC1 activity could alter EV signalling in PCa cells. Inhibition of the AR signalling pathway has also been shown to decrease mTORC1 activity in PCa cells. Moreover, ADT is the standard therapeutic approach for the treatment of men with advanced PCa. Hence, determining whether inhibition of AR signalling alters the type of EVs secreted from PCa cells could have important clinical implications. In addition, it has become increasingly important in the EV field for studies to characterise the type of EVs they have identified to better understand the function of individual EV populations and the signalling pathways underlying their regulation. Therefore, in this chapter, I set out to determine the effects of androgen-deprivation on the type of EVs secreted from PCa cells. I also used mTORC1 inhibition to assess whether anti-androgen treatment produced similar changes in EV signalling to those previously observed, as well as the functional effects of these EVs. Furthermore, I performed detailed characterisation of specific EV cargo in order to identify the vesicle population(s) from which the cargo was derived.

Overall, I found that inhibition of AR signalling in VCaP cells produced changes in EV cargo paralleling those observed in LNCaP cells following mTORC1 inhibition, and that both treatments likely affect more than one EV type. I also found that the AR protein was increased in EVs isolated from both cell lines following these treatments, suggesting that the AR may represent another potential marker of the exosome switch phenotype in PCa cells. Further characterisation revealed that extracellular AR was membrane-enclosed, pelleted in EVs following high-speed centrifugation and partially co-eluted with the exosomal marker syntenin-1 during SEC. Moreover, proliferation assays and gene expression analysis hinted that extracellular AR may also be functional in recipient cells. Altogether these data signify that the AR, or a proportion at least, may be present within exosomes. However, further analysis showed that the majority of extracellular AR, when

separated via SEC, eluted in fractions largely lacking other exosomal markers, except Rab11, suggesting both AR and Rab11 may mark another EV subtype. Overall, these findings indicate that conditions which produce the exosome switch phenotype in PCa cells may regulate a heterogeneous population of EVs, including a subpopulation of vesicles marked by the AR, which could have important effects on recipient cells within the TME.

5.3.1 The AR pathway regulates EV signalling in PCa cells

One of the aims I had beginning this chapter was to investigate the regulatory mechanisms underpinning EV signalling in PCa. I decided to examine whether AR signalling played an important regulatory role in this event by testing the hypothesis that decreased AR signalling promotes the exosome switch phenotype in PCa by inhibiting mTORC1 activity. Overall, I found that the AR pathway is involved in regulating EV signalling in PCa cells, consistent with findings reported in the literature (Soekmadji et al., 2017). Specifically, I showed that inhibition of AR signalling in VCaP cells altered the protein profile of secreted EVs in a manner consistent with the exosome switch observed in LNCaP cells following glutamine depletion. The change in EV cargo, stimulated by bicalutamide treatment, was characterised by an increase in the secretion of Rab11-positive EVs, whereas the level of other EV markers such as syntenin-1, CD63 and CD81 remained unchanged. Such alterations in EV cargo (bar the effect on CD63) are also consistent with those observed in HCT116 cells following PAT4 knockdown and in LNCaP and C4-2B cells following Torin1 treatment, suggesting that the regulatory pathways underpinning these changes may be common among multiple cancer cell types. Moreover, the fact that each of these treatments resulted in decreased mTORC1 activity further implicates mTORC1 as a key regulator of EV signalling in cancer. Although these findings support my hypothesis, further studies are required to determine whether the inhibitory effects of bicalutamide on mTORC1 signalling are necessary to drive the changes in EV cargo observed here. Indeed, the possibility that bicalutamide affects other EV-regulatory pathways cannot be ruled out.

In addition to the characteristic changes in EV signalling that have been described by me in PCa cells, and others in colorectal cancer cells following mTORC1 inhibition (i.e. increased Rab11), I showed that bicalutamide treatment also promoted the secretion of AR-positive EVs from VCaP cells without affecting total AR protein levels. Furthermore, I observed a similar response in the EVs isolated from LNCaP cells following Torin1 treatment and glutamine-depletion, suggesting increased AR may be a novel feature of the exosome switch phenotype in PCa cells.

5.3.2 The AR marks a heterogeneous population of EVs

As discussed in the introduction, the exosome switch phenotype in HCT116 cells involves a change in the type of exosomes that are secreted from cells. Specifically, decreased secretion of exosomes from classic MVEs (i.e. Rab7 positive) and increased secretion from alternative MVEs, marked by Rab11, occurs. In this chapter, I wanted to examine the type of EVs affected during the exosome switching event in PCa cells. I decided to characterise the EV population(s) marked by the AR given that increased secretion of AR-positive EVs appeared characteristic of the exosome switch phenotype in LNCaP and VCaP cells. Furthermore, only very few studies have identified the AR in EVs, hence little is known about the type of the vesicles that contain this cargo or how they may be regulated.

Overall, the AR was detected in EV preps isolated from LNCaP cells via UF, UC and SEC, and from VCaP cells via SEC, supporting the notion that the AR is a genuine marker of EVs in these cells. Moreover, the finding that extracellular AR was resistant to trypsin digestion in the absence of detergent provides further evidence that the AR is present within EVs and not, conversely, a protein contaminant. Indeed, these findings are consistent with two other studies which have similarly reported the AR in EVs isolated from LNCaP cells (Mizutani et al, 2014; Read et al, 2017). However, although both studies detected the AR in EV preps purified via UC, the identity of these AR-positive vesicles was only briefly explored. In this chapter, I found by analysing the SEC elution profile of extracellular AR, that a proportion

of these AR-positive vesicles may be exosomes. For instance, SEC revealed that AR partially co-eluted with syntenin-1, indicating that AR-positive EVs may share similar biophysical characteristics to vesicles marked by syntenin-1 or even be enriched within the same EV subtype. Given that syntenin-1 has been implicated in the biogenesis of exosomes and detected within exosomes isolated from a number of different cell types, these findings lend support to the possibility that AR may also be present within exosome-like vesicles. Further support is provided by the finding that AR also partially co-eluted with Tsg101. Indeed, a defining characteristic of exosomes is that they are formed within MVEs as intraluminal vesicles (ILVs). Considering the formation of ILVs can be regulated by Tsg101 (Bobrie et al., 2011; Raposo and Stoorvogel, 2013), one possibility is that the elution profile observed for Tsg101 is representative of exosomes formed via such ESCRT-dependent pathways. Indeed, CD63 and syntenin-1, EV-associated proteins secreted from MCF-7 and HeLa cells via Tsg101-dependent mechanisms (Baietti et al., 2012; Colombo et al., 2013), were also enriched within these fractions. However, these findings do not rule out the possibility that EV populations other than exosomes also elute here. Indeed, Tsg101 has also been detected in microvesicles (i.e. EVs pelleted at 20,000 *x g*) isolated from LNCaP cells (Saari et al., 2015). Therefore, knockdown studies are ultimately required to determine the route of biogenesis for AR-positive EVs, for example by assessing whether secretion of these vesicles is dependent on certain ESCRT proteins (e.g. Tsg101).

Additional findings presented in this chapter hint that the AR may mark more than one EV population. Using SEC I found that AR-positive EVs predominantly eluted in fractions devoid of other classical exosome markers (i.e. CD63, CD81, syntenin-1 and Tsg101). These fractions were eluted sometime after those enriched for exosomal markers, suggesting that the speed of migration of AR-positive EVs through the SEC column may be slower than classical exosomes, perhaps reflecting the smaller size of these vesicles. Although such a theory could not be confirmed by NTA, it is interesting to note that the tetraspanin proteins (i.e. CD63 and CD81) were not strongly expressed in these fractions

either; perhaps revealing that the EVs eluted here are simply too small to incorporate multiple transmembrane proteins. Interestingly, a novel population of very small particles (<50 nm) termed exomeres were recently identified in EVs isolated from B16-F10 melanoma cells (Zhang et al., 2018). Similar to the population of EVs identified here in the AR-enriched fractions, exomeres lack CD63, CD81 and Tsg101 protein markers. I also observed that the morphology of some particles eluted in these later SEC fractions closely resembled that of exomeres (data not shown). Nonetheless, immuno-EM is necessary to investigate whether AR is actually located within these structures. Although possible, a critical difference between exomeres and the AR-positive vesicles described here, is the authors definition of exomeres as non-membranous nanoparticles. Indeed, this classification is apparently based upon the appearance of these particles visualised by TEM; hence it would be interesting to examine whether exomere cargo is also resistant to trypsin digestion, like the AR. However, another potential difference between exomeres and AR-positive vesicles is the fact that very few Rab proteins have been identified in exomeres, whereas Rab11 was enriched in the same SEC fractions as the AR. Whilst it is possible that Rab11 and AR are not enriched for within the same EV subtype, such an observation does cast doubt as to whether exomeres represent the predominant particle type eluted in these later fractions or if the molecular composition of exomeres simply differs between cell types.

Alternatively to exomeres, other small EV populations have been reported that could also be marked by the AR. For example, the same study that described exomeres also identified two distinct subpopulations of exosomes termed Exo-L (large exosomes, 90-120 nm) and Exo-S (small exosomes, 60-80 nm). Interestingly, the reported size range of the smaller exosome population, Exo-S, falls within the size range of particles predicted to elute in the SEC fractions that I have shown to be predominantly enriched for the AR. Nonetheless, the study further revealed that Exo-S are also enriched for classical exosome markers (i.e. CD63, CD81) as well as multiple components of the ESCRT

machinery, inconsistent with the particles eluted in some of the more AR-rich fractions shown here (i.e. 8-10). Whilst the lack of these markers casts doubt as to whether these AR-positive vesicles are in fact exosomes, the strong expression of Rab11 in the same fractions suggests that endosomal-derived EVs are eluted here. Indeed, whilst in the cell Rab proteins play a central role in defining compartment identity (Chavrier et al., 1990), work completed in HCT116 cells and *Drosophila* has revealed that specific Rabs also serve as molecular signatures for the endocytic origin of EVs (Fan et al., *under revision*). Therefore, the lack of exosome markers and ESCRT proteins, yet enrichment for Rab11 in these AR-enriched fractions, highlights the interesting possibility that the AR may mark a somewhat novel EV type: that is, endosomal in origin, yet distinct from classical exosomes, and formed, perhaps, via ESCRT-independent mechanisms.

Although ESCRT-independent mechanisms of exosome biogenesis have been well documented (Stuffers et al., 2009), others have suggested that the formation of distinct exosomes subpopulations may be regulated by different components of the ESCRT machinery (Maas et al., 2017). Therefore, an alternative explanation for the observed lack of Tsg101 in the AR-enriched fractions could be that EVs marked by the AR are formed via different intracellular mechanisms, but one which may include different ESCRT components. Interestingly, the AR has previously been detected in two distinct exosome populations isolated from LNCaP cells, one expressing CD9 and CD63 together, and the other CD9 alone (Mizutani et al, 2014). Given that the ESCRT-accessory protein Alix has been implicated in the biogenesis of exosomes marked by CD9 but not CD63 (Kowal et al, 2016), it would be interesting to examine the role of Alix in the formation of the AR-positive EVs identified here.

5.3.3 The AR and mTORC1 pathways regulate at least two distinct EV populations

Based upon my analysis of the EV populations altered during the exosome switch event in PCa cells, it appears that a heterogeneous population of EVs may be affected. Indeed, I

found that the AR and Rab11 were increased in all of the SEC fractions examined following mTORC1 inhibition and androgen-deprivation in LNCaP and VCaP cells, respectively. Given that I have also shown the AR elutes in two distinct SEC fractions (i.e. those enriched and non-enriched for classical exosome markers) and is therefore present in at least two different EV subtypes, the non-specific effect of Torin1 and bicalutamide on either population suggests these treatments likely affect the secretion of more than one EV type.

Moreover, the similar elution profiles observed for AR and Rab11 following bicalutamide and Torin1 treatment also suggests that the EV population(s) from which these cargoes are derived may be regulated by similar mechanisms. In addition, the fact that none of the other classical exosome markers were altered by bicalutamide or Torin1 treatment in any of the fractions examined, suggests that the AR and Rab11-positive EVs may represent subpopulations of vesicles within distinct EV populations. Indeed, one intriguing possibility is that the AR is secreted from Rab11-marked compartments, a subcellular location shown in HCT116 cells to lack the classical exosome markers (Fan et al, *under revision*). Further work completed in HCT116 cells has revealed that secretion from Rab11 compartments is regulated by the ESCRT-0 protein STAM1 (Fan et al, *under revision*); hence it would be interesting to examine the effects of STAM1 knockdown on the release of AR-positive vesicles from PCa cells. Overall, my findings suggest that the AR and Rab11 may mark the same or similar type(s) of EV, within at least two distinct EV populations, independent from classical exosomes. Therefore, further analysis is required to determine whether these EVs are derived from endosomal-origins before the phenotype I have characterised in this thesis can truly be termed an ‘exosome’ switch.

5.3.4 Clinical implications

The results presented in this chapter highlight three key findings (i) the AR pathway regulates EV signalling in PCa, (ii) inhibition of AR and mTORC1 signalling promotes secretion of AR-positive EVs from PCa cells and (iii) AR-positive vesicles represent a

heterogenous population of EVs. Overall, these findings provide important insights into the regulatory mechanisms underpinning EV signalling in PCa cells as well as the molecular and biophysical characteristics of EVs secreted in clinical settings relevant to PCa progression. Considering there is significant interest in developing EV-based diagnostic, prognostic and therapeutic applications, such insights could have important implications.

In terms of clinical applications of EVs in PCa, it is hoped that EVs could eventually act as biomarkers to aid in cancer diagnosis and monitor treatment response. Currently, the diagnostic and prognostic methods available, fine-needle biopsy for example, frequently result in significant patient morbidity due to their invasive nature. EVs, on the other hand, can be isolated with relative ease from a variety of different biological fluids and could potentially offer a non-invasive liquid biopsy. In addition, it has recently been shown that PSA measurements alone are unable to effectively monitor disease activity in advanced CRPC (Pezaro et al., 2014). Therefore, it has been suggested that EVs could replace, or be used alongside, PSA measurements to determine patient prognosis. However, as yet, no such EV-derived biomarker indicative of treatment response has been identified in PCa.

In this chapter, I found that the AR pathway is involved in regulating EV cargo in VCaP cells. Whilst recent studies have also hinted at a role for the AR in EV signalling in PCa (Soekmadji et al., 2017; Peak et al., 2017), my work is the first to suggest crosstalk between AR and mTORC1 as the regulatory mechanism underpinning this control. As discussed in the previous chapter, alterations in the PI3K/Akt/mTOR pathway are frequent in patients with advanced PCa and implicated in the development of CRPC. Therefore, further characterisation of EV cargo secreted from PCa cells following androgen-deprivation, with and without mTOR inhibitors, may enable a specific EV signature to be identified. For instance, based upon my findings, I hypothesise that the exosome switch phenotype is observed only when mTORC1 activity is low. Therefore, examining EV cargo prior to and during treatment could enable the level of mTORC1 activity in the secreting PCa cells to be

inferred. Indeed, using such a strategy, responsiveness to ADT could be monitored, whilst patients requiring further intervention (e.g. mTOR inhibitors) could also be identified.

Although EVs may offer a potential source of biomarkers for the diagnosis of PCa and monitoring of disease progression, they also hold great potential as therapeutic targets themselves. Indeed, EVs are regulated by a variety of stimuli such as hypoxia, nutrient starvation and anti-cancer therapies (Parolini et al., 2009; Kucharzewska et al., 2013; Azmi et al., 2014), and EVs derived from PCa cells have been implicated in driving a host of processes involved in facilitating primary and secondary tumour growth, including cell proliferation (Soekmadji et al., 2016), angiogenesis (Naito et al., 2017), metastasis (Bijnsdorp et al., 2013; Liu et al., 2016), and immune evasion (Lundholm et al., 2014). As such, there is considerable interest in targeting EVs to prevent PCa progression.

The data I have presented here indicates that inhibition of either AR or mTORC1 signalling increases the secretion of AR-positive EVs from PCa cells. Furthermore, I have shown that EVs derived from mTORC1-inhibited cells can promote PCa cell proliferation and the expression of AR-regulated genes in recipient cells. Although additional analysis is required to determine whether the AR is responsible for driving such functional effects, these findings are consistent with another study that reported the AR and the mutant variant, AR-V7, are secreted in EVs from PCa cells and can be transported to the nucleus of prostate cells *in vivo* (Read et al., 2016). Together, these findings highlight the interesting possibility that PCa cells may employ horizontal transfer of the AR to cells within the TME to overcome ADT or other environmental stresses. Indeed, as a mechanism, increasing the level of AR protein is a known driver of CRPC by amplifying signal output from low levels of androgens and altering the cells response to antagonists (Chen et al., 2004). Furthermore, a recent study showed that EVs isolated from enzalutamide-resistant C4-2B cells contained increased levels of AR-V7 compared to their enzalutamide-sensitive counterparts (Peak et al., 2017). Likewise, the level of AR-V7 RNA in EVs derived from the plasma of PCa patients

has recently been highlighted as a predictive biomarker of resistance to hormone therapy (Del Re et al., 2017). Overall, these studies support my own findings that anti-androgen treatment enhances the secretion of AR-positive EVs from PCa cells, and potentially reveals this to be a common mechanism underlying drug-resistance in PCa. Nonetheless, to target such a pathway, the specific EV types responsible, and their routes of biogenesis must first be characterised.

My findings suggest that the AR marks at least two different EV populations in PCa cells. Although my data further indicates that anti-androgen treatment similarly affects both populations, it is possible that the AR within these two EV subtypes regulates different biological processes. So far, I have examined the functional effects of a mixed EV population on AR-regulated cellular events (i.e. transcription, proliferation). Therefore, in the future, it will be important to assess the effects of individual AR-positive EV populations in target cells. Indeed, some of my preliminary data suggests that EVs derived from two distinct AR-positive SEC fractions may differentially regulate PCa cell growth. Therefore, we must fully characterise the functional differences between these different EV subtypes in order to identify appropriate targets for therapeutic intervention.

CHAPTER 6

CONCLUSIONS

6. Concluding remarks

6.1 Summary of research aims

Caring for patients with PCa is one of the major global healthcare challenges facing us today. Given the world's population is ageing at an accelerated rate (He et al., 2016) and the diagnosis of PCa is becoming increasingly frequent (Mayor et al., 2012), developing strategies to treat this disease will only become more important in the future. Despite recent improvements in treatment, advanced PCa continues to be associated with a high rate of mortality (Kelly et al., 2018). Therefore, the clinical aim I wanted to address in my research was to understand the ways in which intercellular signalling mechanisms regulate PCa cell growth and, in turn, highlight novel targets for the treatment of aggressive PCa.

In recent years, the TME has been revealed as a key participant in PCa progression and, therefore, a potentially exciting therapeutic target. Indeed, for PCa to advance, cancer cells must communicate with other components in the TME. Hence, strategies aimed at interrupting such interactions could provide potent anti-cancer therapies. Therefore, I decided to focus my doctoral research on investigating the ways in which certain microenvironmental signals regulate PCa progression, specifically those involving BMP and mTORC1 signalling, for reasons discussed in the introduction. Considering I have explored the clinical implications of my work throughout, in this chapter I summarise my main research findings in relation to the aims stated at the beginning of this thesis. To recap, these are as follows:

Aims:

i. Characterise the effects of BMP signalling on PCa cell behaviour
ii. Determine the role of the PAT4-mTORC1 signalling axis in PCa cells
iii. Establish whether the mTORC1 pathway regulates distinct EV subtypes

6.1.1 Aim (i): BMP-6 inhibits the growth of androgen-sensitive PCa cells

Although much work has been done to understand the role of BMP-6 in PCa progression, particularly cell growth, conflicting findings due to differing culture conditions have meant that very few conclusions have yet been drawn. Therefore, one of the initial objectives I had was to perform a growth screen in a range of culture conditions to determine the effect of BMP-6 on PCa cell growth. Overall, this screen revealed that BMP-6 stimulates a largely growth-inhibitory response in LNCaP cells. However, I am aware that some of my data conflicts with other published findings. Although differing culture conditions are likely responsible, these discrepancies have caused me to question the validity of my own findings and whether I have utilised the 'correct' culture conditions in my work. Whilst these concerns further highlight to me the limitations of investigating the role of the TME *in vitro*, the strong inhibitory response I observed in LNCaP cell proliferation when co-cultured with the bone stromal cell lines, increases my confidence in my hypothesis that the bone environment is normally growth-suppressive in PCa.

Initially, I was confused by the notion that BMP-6 may be acting to inhibit PCa cell proliferation in the bone environment, given that BMP-6 expression is enhanced in advanced PCa (Hamdy et al, 1997; Autzen et al, 1998). However, upon learning that several other BMPs have been implicated in driving the process of metastatic dormancy (Sosa et al, 2014), I now wonder whether BMP-6 could serve a similar role in bone-metastasised PCa. Indeed, tumour dormancy is believed to be a common event in men with recurrent PCa (Morgan et al, 2009), and one which is supposedly regulated by growth-inhibitory signals in the bone marrow (Shiozawa et al, 2011). Hence, BMP-6 may represent one such signal. Ultimately however, I am convinced that *in vivo* studies are necessary if we are to unpick the role of BMP-6 in regulating PCa cell growth in the context of the bone environment.

6.1.2 Aim (I): AR and Rb may mediate the growth-inhibitory effects of BMP-6

Having established that BMP-6 inhibits the growth of LNCaP cells *in vitro*, I began a series of experiments aimed at uncovering the regulatory mechanisms underpinning this effect. Several observations compelled me to pursue the theory that the AR pathway is involved in mediating the growth-suppressive effects of BMP-6. Ultimately, I found that BMP-6-induced growth inhibition is associated with reduced AR activity and decreased Rb phosphorylation. Having also discovered that loss of Rb diminished the suppressive effects of BMP-6 on LNCaP cell growth, I am keen to see whether future studies will implicate a role for the newly-identified, Rb-dependent, anti-proliferative function of AR in this event (Gao et al, 2016).

Therefore, regarding aim (i), I have characterised the effect of BMP-6 on PCa cell growth and proposed a working model to explain my observations in light of existing clinical data. Overall, I speculate that loss of Rb and/or alterations in AR signalling, both of which are commonly observed in advanced PCa, enables cells to overcome the growth-inhibitory effects of BMP-6 in the bone environment, leading to aggressive disease. Indeed, such a concept is not unlike that which has already been proposed to explain metastatic relapse in breast cancer, where suppression of BMP signalling drives the reactivation of dormant breast cancer cells in the lung (Gao et al., 2012). Intriguingly, BMP signalling has also been shown to inhibit breast cancer cell growth and Rb phosphorylation *in vitro* (Ghosh-Choudhury et al., 2000), suggesting that a parallel mechanism may underpin the growth-inhibitory effects of BMPs in other cancer cell types. Indeed, related findings have also been reported for thyroid carcinoma cells (Franzén et al., 2001). Considering the cancer cell types in which these effects have so far been observed include those derived from adenocarcinomas, particularly tissues whose growth is normally under hormonal control, raises the exciting possibility that crosstalk between BMP and the Rb/hormone receptor signalling axis may represent a common pathway underpinning cell growth in hormone-

driven cancers. Indeed, such a theory could be explored further by examining whether the mechanisms identified here in PCa cells exist in these other cancer cell types.

6.1.3 Aim (ii): PAT4 regulates mTORC1 activity and PCa cell growth

The AR signalling pathway plays a key role in regulating normal prostate growth and prostate carcinogenesis (Koivisto et al., 1998; Lin et al.,1998; Abate-Shen et al., 2000; Nantermet et al., 2004). Before beginning my doctoral studies, work completed by another student in the host lab revealed that expression of the amino acid transporter PAT4 was enhanced in malignant prostate tissue (Dan Stevens, unpublished). Therefore, one theory I wanted to test during my DPhil was that androgens drive PAT4 expression. Given my findings in VCaP cells support such a theory, I now propose PAT4 to be among the amino acid transporters positively regulated by the AR to mediate the effects of androgens on PCa cell growth (Lao et al., 1993; Xu et al., 2006; Wang et al., 2011). Although I was unable to investigate the mechanisms underlying these functional effects in my work, further experiments may provide insight into a potential transceptor role for PAT4 given its cytoplasmic localisation in PCa tissue.

My findings also raise the interesting possibility that PAT4 expression and function may be regulated by similar mechanisms in other hormone-driven cancers, such as breast cancer. Previous work in our lab has shown that PAT4 positively regulates growth and mTORC1 signalling in breast cancer cells (Heublein et al., 2010), although the influence of estrogens has not yet been examined. Estrogens have, however, been shown to alter the expression of several other members of the SLC transporter family. For example, SLC1A5 and SLC6A14, two glutamine-sensitive transporters, are upregulated in breast cancer (Karunakaran et al., 2011; Kim et al., 2013) and positively regulated by the estrogen receptor (ER) pathway (Todorova et al., 2011). One intriguing possibility is that the hormone-driven expression of amino acid transporters, including PAT4, reflects an important regulatory mechanism which normally controls cell proliferation during

development, and becomes reactivated to enhance tumour growth during oncogenic transformation.

Curiously however, whilst changes in *PAT4* mRNA expression are significantly more common in metastatic than localised PCa cases, most of these changes appear to be reductions (Taylor et al., 2010). Given that AR signalling is typically enhanced in advanced PCa (Koivisto et al., 1998), and I have shown the expression of *PAT4* mRNA is upregulated by androgens, these clinical data potentially cast doubt upon my own findings. It would therefore be interesting to investigate whether the metastatic samples that exhibited reduced PAT4 levels were derived from patients undergoing hormone-deprivation therapy. If so, these findings may suggest instead that AR inhibition is associated with reduced PAT4 expression, supporting my hypothesis that androgen signalling normally enhances PAT4 protein levels in PCa.

6.1.4 Aim (ii): mTORC1 signalling regulates EV signalling in PCa cells

Recent work completed in our lab has revealed that Rab11-marked endocytic compartments contain an alternative source of exosomes. Moreover, additional findings have suggested that these 'switched' exosomes are preferentially secreted from colorectal cancer cells in conditions of nutrient stress to promote pro-tumorigenic functions in recipient cells (Fan et al, *in prep*). My findings indicate that the PAT4-mTORC1 signalling axis may regulate a similar switching event in PCa. Indeed, I too have shown that EVs derived from mTORC1-inhibited cells can drive cancer cell growth. Analogous findings have now been reported for several other cancer cell lines (Shih Jung Fan and John Mason; unpublished data), suggesting that secretion of these alternative EVs may represent a common adaptive response employed by many types of cancer to overcome metabolic stress in the TME. Future work should address whether non-malignant cells are also able to regulate EV secretion in this way, or if cells acquire such abilities during oncogenic reprogramming.

Interestingly, studies performed by our collaborators have demonstrated that (i) exosomes can form within Rab11-positive compartments (Ben Kroeger, Wilson lab; unpublished data) and (ii) that exosome secretion is regulated by mTOR-dependent mechanisms (Aaron Leiblich, Wilson lab; unpublished data) in cells within the *Drosophila* accessory gland. Considering the accessory gland shares many features with, and functions similarly to, the mammalian prostate (Leiblich et al., 2012), these findings indicate that the regulatory mechanisms underlying EV signalling in PCa may be conserved. Moving forwards, it will be important to identity further parallels between these two systems as well as any distinguishing features which, together, could provide key insights into the aspects of cell biology that may have gone awry in PCa.

Therefore, regarding aim (ii), I have shown that secreted factors (i.e. androgens) can drive PCa cell growth by enhancing the expression of upstream components (i.e. PAT4) of the mTORC1 signalling pathway. In addition to enhancing cell growth via intracellular signalling mechanisms, I have provided evidence to suggest that the PAT4-mTORC1 axis may also regulate PCa cell growth via intercellular signalling routes (i.e. EVs), in response to changing levels of nutrients in the TME.

6.1.5 Aim (iii): Microenvironmental stress promotes exosome switching in PCa cells

The work that has been completed so far in our lab by myself and others has focused on characterising changes in EV signalling in response to nutrient stress-induced mTORC1 inhibition. However, mTORC1 signalling is regulated by numerous extracellular stimuli. In PCa, androgens enhance mTORC1 signalling (Xu et al., 2006; Rai et al., 2010; Tsouko et al., 2014); conversely, androgen-deprivation inhibits mTORC1 activity (Wu et al., 2010). Therefore, I wanted to test whether blocking AR activity could also regulate EV signalling in PCa cells. My findings indicate that androgen-deprivation promotes similar changes in EV cargo to mTORC1 inhibition, highlighting that alterations in extracellular conditions other than nutrient availability also regulate the secretion of these alternative EVs. Therefore, to

revise my original hypothesis, I propose that the secretion of 'switched' EVs may be an adaptive response employed by tumour cells to combat *mTORC1-induced* microenvironmental stress. Furthermore, these findings also raise the interesting possibility that the inhibitory effects of anti-androgens on PAT4 expression may underpin the changes in EV signalling. However, further experiments are necessary to fully establish the effects of androgen-deprivation on PAT4, and the role of PAT4 in EV secretion.

My work now paves the way for further studies to investigate the effects of other environmental stress signals on the process of exosome switching in PCa. Indeed, mTORC1 activity is regulated by multiple extracellular inputs. In addition to amino acids, nutrients such as glucose and oxygen also modulate mTORC1 activity (Dibble et al., 2013), and are often limited within the TME. However, I personally would be interested in examining the effects of BMP signalling in this process. In PCa, BMP signalling is normally suppressed by mTORC1 activity, although deregulation has been linked to disease progression (Wahdan-Alaswad et al., 2012). Furthermore, BMP signalling has been shown to regulate exosome secretion in the *Drosophila* accessory gland (Corrigan et al., 2014). Hence, one intriguing possibility is that the effects of mTORC1 inhibition on EV secretion are mediated by the BMP pathway. Further experiments could expand upon my own work by assessing whether hyperactive BMP signalling promotes exosome switching in PCa cells, providing additional insights into the role(s) this pathway may play in PCa progression.

6.1.6 Aim (iii): Switched EVs include a heterogeneous population of EVs

Vesicles formed within, and secreted from, Rab11-marked compartments are proposed to represent an alternative type of exosome (Fan, Kroeger et al, *in prep*); however, very little is known about this novel EV population. For example: are Rab11-positive vesicles derived from one single EV population or do multiple EV subtypes exist? If so; which populations are regulated by mTORC1 signalling? Do their functions differ? My findings revealed that, in addition to Rab11, PCa cells secrete a greater proportion of AR-positive EVs as part of

their ‘switching’ response. These EVs, along with Rab11-positive vesicles, represent at least two different EV subtypes and the secretion of each is enhanced during the ‘exosome switching’ event. One intriguing possibility is that, in response to microenvironmental stress, PCa cells employ EVs to mediate horizontal transfer of the AR to neighbouring tumour cells, driving their oncogenic reprogramming and disease progression. Whilst my findings hint that the AR secreted within EVs may be functional in recipient cells, knockdown studies are critical to provide the evidence in favour of such a mechanism.

Importantly, these findings also have significant implications for future EV studies. As a technique, SEC is becoming increasingly popular as the method of choice for EV isolation. However, most of the studies aimed at characterising the exosomal content of SEC fractions have focused on defining the elution profiles of tetraspanins (Böing et al., 2014; Gámez-Valero et al., 2016; Montaner-Tarbes et al., 2016; Benedikter et al., 2017) and ESCRT proteins (Baranyai et al., 2015). Consequently, resultant protocols for EV isolation are devised based upon these findings (Corso et al., 2017). However, my work is the first to reveal that another exosomal marker of endocytic origin, Rab11, is enriched in the SEC fractions largely devoid of these classical exosome markers. Therefore, it is possible that the exosome populations eluted in these later fractions are excluded from further analysis due to the current SEC protocols. Given that these EVs may serve important functional roles in health and disease, future studies should consider examining these fractions to ensure a comprehensive EV analysis.

Therefore, regarding aim (iii), I have shown that changes in the microenvironment that affect mTORC1 activity, in addition to metabolic stress, also regulate EV signalling in PCa. Furthermore, I have revealed that alterations in EV cargo, catalysed by microenvironmental stress conditions, potentially reflect increased secretion of both classical and non-classical exosome populations. Finally, I propose that these EVs may support PCa cell propagation in the TME via the transfer of oncogenic cargoes.

7. References

Aalberts, M., van Dissel-Emiliani, F. M., van Adrichem, N. P., van Wijnen, M., Wauben, M. H., Stout, T. A. and Stoorvogel, W. (2012) 'Identification of distinct populations of prostasomes that differentially express prostate stem cell antigen, annexin A1, and GLIPR2 in humans', *Biol Reprod,* 86(3), pp. 82.

Abate-Shen, C. and Shen, M. M. (2000) 'Molecular genetics of prostate cancer', *Genes Dev,* 14(19), pp. 2410-34.

Ablin, R. J. and Farnsworth, W. E. (1989) *The Prostate as an Endocrine Gland.* Taylor & Francis.

Aggarwal, R. R., Alumkal, J. J., Szmulewitz, R. Z., Higano, C. S., Bryce, A. H., Yu, M. K., De Boer, C., Choque-Gonzales, G. and Small, E. J. (2015) 'The role of highly selective androgen receptor (AR) targeted therapy in men with biochemically relapsed hormone sensitive prostate cancer', *Journal of Clinical Oncology,* 33(15_suppl), pp. TPS5084-TPS5084.

Aitchison, A. A., Veerakumarasivam, A., Vias, M., Kumar, R., Hamdy, F. C., Neal, D. E. and Mills, I. G. (2008) 'Promoter methylation correlates with reduced Smad4 expression in advanced prostate cancer', *Prostate,* 68(6), pp. 661-74.

Amling, C. L., Blute, M. L., Bergstralh, E. J., Seay, T. M., Slezak, J. and Zincke, H. (2000) 'Long-term hazard of progression after radical prostatectomy for clinically localized prostate cancer: continued risk of biochemical failure after 5 years', *J Urol,* 164(1), pp. 101-5.

Armstrong, A. J., Netto, G. J., Rudek, M. A., Halabi, S., Wood, D. P., Creel, P. A., Mundy, K., Davis, S. L., Wang, T., Albadine, R., Schultz, L., Partin, A. W., Jimeno, A., Fedor, H., Febbo, P. G., George, D. J., Gurganus, R., De Marzo, A. M. and Carducci, M. A. (2010) 'A Pharmacodynamic Study of Rapamycin in Men with Intermediate- to High-Risk Localized Prostate Cancer', *Clinical Cancer Research,* 16(11), pp. 3057-3066.

Armstrong, A. J., Shen, T., Halabi, S., Kemeny, G., Bitting, R. L., Kartcheske, P., Embree, E., Morris, K., Winters, C., Jaffe, T., Fleming, M. and George, D. J. (2013) 'A Phase II Trial of Temsirolimus in Men With Castration-Resistant Metastatic Prostate Cancer', *Clinical Genitourinary Cancer,* 11(4), pp. 397-406.

Autzen, P., Robson, C. N., Bjartell, A., Malcolm, A. J., Johnson, M. I., Neal, D. E. and Hamdy, F. C. (1998) 'Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies', *British Journal Of Cancer,* 78, pp. 1219.

Arora, M. (2013) ' Cell culture media: A review'. *Mater methods*, 3, pp.175

Azmi, A. S., Bao, B., & Sarkar, F. H. (2013). 'Exosomes in Cancer Development, Metastasis and Drug Resistance: A Comprehensive Review', *Cancer Metastasis Reviews*, *32*(0), pp. 10.1007/s10555–013–9441–9.

Babst, M. (2011) 'MVB Vesicle Formation: ESCRT-Dependent, ESCRT-Independent and Everything in Between', *Current opinion in cell biology,* 23(4), pp. 452-457.

Babst, M., Katzmann, D. J., Estepa-Sabal, E. J., Meerloo, T. and Emr, S. D. (2002) 'Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting', *Dev Cell,* 3(2), pp. 271-82.

Baietti, M. F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E., Zimmermann, P. and David, G. (2012) 'Syndecan-syntenin-ALIX regulates the biogenesis of exosomes', *Nat Cell Biol,* 14(7), pp. 677-85.

Bar-Peled, L., Chantranupong, L., Cherniack, A. D., Chen, W. W., Ottina, K. A., Grabiner, B. C., Spear, E. D., Carter, S. L., Meyerson, M. and Sabatini, D. M. (2013) 'A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1', *Science,* 340(6136), pp. 1100-6.

Bar-Peled, L., Schweitzer, L. D., Zoncu, R. and Sabatini, D. M. (2012) 'Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1', *Cell,* 150(6), pp. 1196-208.

Baranyai, T., Herczeg, K., Onódi, Z., Voszka, I., Módos, K., Marton, N., Nagy, G., Mäger, I., Wood, M. J., El Andaloussi, S., Pálinkás, Z., Kumar, V., Nagy, P., Kittel, Á., Buzás, E. I., Ferdinandy, P. and Giricz, Z. (2015) 'Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods', *PLOS ONE,* 10(12), pp. e0145686.

Barnes, J., Anthony, C. T., Wall, N. and Steiner, M. S. (1995) 'Bone morphogenetic protein-6 expression in normal and malignant prostate', *World J Urol,* 13(6), pp. 337-43.

Ben-Shlomo, Y., Evans, S., Ibrahim, F., Patel, B., Anson, K., Chinegwundoh, F., Corbishley, C., Dorling, D., Thomas, B., Gillatt, D., Kirby, R., Muir, G., Nargund, V., Popert, R., Metcalfe, C. and Persad, R. (2008) 'The risk of prostate cancer amongst black men in the United Kingdom: the PROCESS cohort study', *Eur Urol,* 53(1), pp. 99-105.

Benedikter, B. J., Bouwman, F. G., Vajen, T., Heinzmann, A. C. A., Grauls, G., Mariman, E. C., Wouters, E. F. M., Savelkoul, P. H., Lopez-Iglesias, C., Koenen, R. R., Rohde, G. G. U. and Stassen, F. R. M. (2017) 'Ultrafiltration combined with size exclusion chromatography efficiently isolates extracellular vesicles from cell culture media for compositional and functional studies', *Scientific Reports,* 7(1), pp. 15297.

Bentley, H., Hamdy, F. C., Hart, K. A., Seid, J. M., Williams, J. L., Johnstone, D. and Russell, R. G. (1992) 'Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia', *British Journal of Cancer,* 66(6), pp. 1159-1163.

Bijnsdorp, I. V., Geldof, A. A., Lavaei, M., Piersma, S. R., van Moorselaar, R. J. A. and Jimenez, C. R. (2013) 'Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients', *Journal of Extracellular Vesicles,* 2, pp. 10.3402/jev.v2i0.22097.

Bill-Axelson, A., Holmberg, L., Garmo, H., Rider, J. R., Taari, K., Busch, C., Nordling, S., Haggman, M., Andersson, S. O., Spangberg, A., Andren, O., Palmgren, J., Steineck, G., Adami, H. O. and Johansson, J. E. (2014) 'Radical prostatectomy or watchful waiting in early prostate cancer', *N Engl J Med,* 370(10), pp. 932-42.

Blum, B., Moseley, J., Miller, L., Richelsoph, K. and Haggard, W. (2004) 'Measurement of bone morphogenetic proteins and other growth factors in demineralized bone matrix', *Orthopedics,* 27(1 Suppl), pp. s161-5.

Bobrie, A., Colombo, M., Raposo, G. and Théry, C. (2011) 'Exosome Secretion: Molecular Mechanisms and Roles in Immune Responses', *Traffic,* 12(12), pp. 1659-1668.

Bobrie, A., Krumeich, S., Reyal, F., Recchi, C., Moita, L. F., Seabra, M. C., Ostrowski, M. and Thery, C. (2012) 'Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression', *Cancer Res,* 72(19), pp. 4920-30.
Böing, A. N., van der Pol, E., Grootemaat, A. E., Coumans, F. A. W., Sturk, A. and Nieuwland, R. (2014) 'Single-step isolation of extracellular vesicles by size-exclusion chromatography', *Journal of Extracellular Vesicles,* 3, pp. 10.3402/jev.v3.23430.

Bubendorf, L., Schopfer, A., Wagner, U., Sauter, G., Moch, H., Willi, N., Gasser, T. C. and Mihatsch, M. J. (2000) 'Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients', *Hum Pathol,* 31(5), pp. 578-83.

Buschow, S. I., Liefhebber, J. M. P., Wubbolts, R. and Stoorvogel, W. (2005) 'Exosomes contain ubiquitinated proteins', *Blood Cells, Molecules, and Diseases,* 35(3), pp. 398-403.

Buschow, S. I., Nolte-'t Hoen, E. N., van Niel, G., Pols, M. S., ten Broeke, T., Lauwen, M., Ossendorp, F., Melief, C. J., Raposo, G., Wubbolts, R., Wauben, M. H. and Stoorvogel, W. (2009) 'MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways', *Traffic,* 10(10), pp. 1528-42.

Byrne, R. L., Leung, H. and Neal, D. E. (1996) 'Peptide growth factors in the prostate as mediators of stromal epithelial interaction', *British Journal of Urology,* 77(5), pp. 627-633.

Carver, B. S., Chapinski, C., Wongvipat, J., Hieronymus, H., Chen, Y., Chandarlapaty, S., Arora, V. K., Le, C., Koutcher, J., Scher, H., Scardino, P. T., Rosen, N. and Sawyers, C. L. (2011) 'Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer', *Cancer Cell,* 19(5), pp. 575-86.

Caubet, J.-F., Tosteson, T. D., Dong, E. W., Naylon, E. M., Whiting, G. W., Ernstoff, M. S. and Ross, S. D. (1997) 'Maximum androgen blockade in advanced prostate cancer: A meta-analysis of published randomized controlled trials using nonsteroidal antiandrogens', *Urology,* 49(1), pp. 71-78.

Chang, K. H., Ercole, C. E. and Sharifi, N. (2014) 'Androgen metabolism in prostate cancer: from molecular mechanisms to clinical consequences', *British Journal of Cancer,* 111(7), pp. 1249-1254.

Chantranupong, L., Wolfson, R. L., Orozco, J. M., Saxton, R. A., Scaria, S. M., Bar-Peled, L., Spooner, E., Isasa, M., Gygi, S. P. and Sabatini, D. M. (2014) 'The Sestrins interact with GATOR2 to negatively regulate the amino acid sensing pathway upstream of mTORC1', *Cell reports,* 9(1), pp. 1-8.

Chavrier, P., Vingron, M., Sander, C., Simons, K. and Zerial, M. (1990) 'Molecular cloning of YPT1/SEC4-related cDNAs from an epithelial cell line', *Molecular and Cellular Biology,* 10(12), pp. 6578-6585.

Chen, D., Zhao, M. and Mundy, G. R. (2004) 'Bone morphogenetic proteins', *Growth Factors,* 22(4), pp. 233-41.

Chen, S., Wang, J., Yu, G., Liu, W. and Pearce, D. (1997) 'Androgen and glucocorticoid receptor heterodimer formation. A possible mechanism for mutual inhibition of transcriptional activity', *J Biol Chem,* 272(22), pp. 14087-92.

Chen, Y.-W., Lee, M.-S., Lucht, A., Chou, F.-P., Huang, W., Havighurst, T. C., Kim, K., Wang, J.-K., Antalis, T. M., Johnson, M. D. and Lin, C.-Y. (2010) 'TMPRSS2, a Serine Protease Expressed in the Prostate on the Apical Surface of Luminal Epithelial Cells and Released into Semen in Prostasomes, Is Misregulated in Prostate Cancer Cells', *The American Journal of Pathology,* 176(6), pp. 2986-2996.
Chmelar, R., Buchanan, G., Need, E. F., Tilley, W. and Greenberg, N. M. (2007) 'Androgen receptor coregulators and their involvement in the development and progression of prostate cancer', *Int J Cancer,* 120(4), pp. 719-33.

Choo, A. Y., Yoon, S.-O., Kim, S. G., Roux, P. P. and Blenis, J. (2008) 'Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation', *Proceedings of the National Academy of Sciences of the United States of America,* 105(45), pp. 17414-17419.

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L. F., Thery, C. and Raposo, G. (2013) 'Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles', *J Cell Sci,* 126(Pt 24), pp. 5553-65.

Colombo, M., Raposo, G. and Thery, C. (2014) 'Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles', *Annu Rev Cell Dev Biol,* 30, pp. 255-89.

Cook, M. B., Rosenberg, P. S., McCarty, F. A., Wu, M., King, J., Eheman, C. and Anderson, W. F. (2015) 'Racial disparities in prostate cancer incidence rates by census division in the United States, 1999–2008', *The Prostate,* 75(7), pp. 758-763.

Corcoran, C., Rani, S., O'Brien, K., O'Neill, A., Prencipe, M., Sheikh, R., Webb, G., McDermott, R., Watson, W., Crown, J. and O'Driscoll, L. (2012) 'Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes', *PLoS One,* 7(12), pp. e50999.

Corrigan, L., Redhai, S., Leiblich, A., Fan, S. J., Perera, S. M., Patel, R., Gandy, C., Wainwright, S. M., Morris, J. F., Hamdy, F., Goberdhan, D. C. and Wilson, C. (2014) 'BMP-regulated exosomes from Drosophila male reproductive glands reprogram female behavior', *J Cell Biol,* 206(5), pp. 671-88.

Corso, G., Mäger, I., Lee, Y., Görgens, A., Bultema, J., Giebel, B., Wood, M. J. A., Nordin, J. Z. and Andaloussi, S. E. L. (2017) 'Reproducible and scalable purification of extracellular vesicles using combined bind-elute and size exclusion chromatography', *Scientific Reports,* 7(1), pp. 11561.

Crawford, E. D., Eisenberger, M. A., McLeod, D. G., Spaulding, J. T., Benson, R., Dorr, F. A., Blumenstein, B. A., Davis, M. A. and Goodman, P. J. (1989) 'A controlled trial of leuprolide with and without flutamide in prostatic carcinoma', *N Engl J Med,* 321(7), pp. 419-24.

Crowe, F. L., Allen, N. E., Appleby, P. N., Overvad, K., Aardestrup, I. V., Johnsen, N. F., Tjonneland, A., Linseisen, J., Kaaks, R., Boeing, H., Kroger, J., Trichopoulou, A., Zavitsanou, A., Trichopoulos, D., Sacerdote, C., Palli, D., Tumino, R., Agnoli, C., Kiemeney, L. A., Bueno-de-Mesquita, H. B., Chirlaque, M. D., Ardanaz, E., Larranaga, N., Quiros, J. R., Sanchez, M. J., Gonzalez, C. A., Stattin, P., Hallmans, G., Bingham, S., Khaw, K. T., Rinaldi, S., Slimani, N., Jenab, M., Riboli, E. and Key, T. J. (2008) 'Fatty acid composition of plasma phospholipids and risk of prostate cancer in a case-control analysis nested within

the European Prospective Investigation into Cancer and Nutrition', *Am J Clin Nutr,* 88(5), pp. 1353-63.

Culig, Z., Hobisch, A., Cronauer, M. V., Cato, A. C., Hittmair, A., Radmayr, C., Eberle, J., Bartsch, G. and Klocker, H. (1993) 'Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone', *Molecular Endocrinology,* 7(12), pp. 1541-1550.

Culig, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G. and Klocker, H. (1994) 'Androgen Receptor Activation in Prostatic Tumor Cell Lines by Insulin-like Growth Factor-I, Keratinocyte Growth Factor, and Epidermal Growth Factor', *Cancer Research,* 54(20), pp. 5474-5478.

Cunha, G. R. and Donjacour, A. A. (1989) 'Mesenchymal-epithelial interactions in the growth and development of the prostate', *Cancer Treat Res,* 46, pp. 159-75.

Dada, S., Demartines, N. and Dormond, O. (2008) 'mTORC2 regulates PGE2-mediated endothelial cell survival and migration', *Biochemical and Biophysical Research Communications,* 372(4), pp. 875-879.

Dai, J., Hall, C. L., Escara-Wilke, J., Mizokami, A., Keller, J. M. and Keller, E. T. (2008) 'Prostate cancer induces bone metastasis through Wnt-induced bone morphogenetic protein-dependent and independent mechanisms', *Cancer research,* 68(14), pp. 5785-5794.

Dai, J., Keller, J., Zhang, J., Lu, Y., Yao, Z. and Keller, E. T. (2005) 'Bone morphogenetic protein-6 promotes osteoblastic prostate cancer bone metastases through a dual mechanism', *Cancer Res,* 65(18), pp. 8274-85.

Dammai, V. (2011) 'Endosomal Compartments', in Schwab, M. (ed.) *Encyclopedia of Cancer*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 1244-1248.

Darby, S., Cross, S. S., Brown, N. J., Hamdy, F. C. and Robson, C. N. (2008) 'BMP-6 over-expression in prostate cancer is associated with increased Id-1 protein and a more invasive phenotype', *J Pathol,* 214(3), pp. 394-404.

Dasgupta, S., Putluri, N., Long, W., Zhang, B., Wang, J., Kaushik, A. K. and O'Malley, B. W. (2015). 'Coactivator SRC-2–dependent metabolic reprogramming mediates prostate cancer survival and metastasis', *The Journal of Clinical Investigation*, *125*(3), pp. 1174–1188.

Deatherage, B. L. and Cookson, B. T. (2012) 'Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life', *Infect Immun,* 80(6), pp. 1948-57.

Dehm, S. M. and Tindall, D. J. (2011) 'Alternatively spliced androgen receptor variants', 18(5), pp. R183.

Del Re, M., Biasco, E., Crucitta, S., Derosa, L., Rofi, E., Orlandini, C., Miccoli, M., Galli, L., Falcone, A., Jenster, G. W., van Schaik, R. H. and Danesi, R. (2017) 'The Detection of Androgen Receptor Splice Variant 7 in Plasma-derived Exosomal RNA Strongly Predicts Resistance to Hormonal Therapy in Metastatic Prostate Cancer Patients', *Eur Urol,* 71(4), pp. 680-687.

DePrimo, S. E., Diehn, M., Nelson, J. B., Reiter, R. E., Matese, J., Fero, M., Tibshirani, R., Brown, P. O. and Brooks, J. D. (2002) 'Transcriptional programs activated by exposure of human prostate cancer cells to androgen', *Genome Biology,* 3(7), pp. research0032.1-research0032.12.

DeYoung, M. P., Horak, P., Sofer, A., Sgroi, D. and Ellisen, L. W. (2008) 'Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling', *Genes Dev,* 22(2), pp. 239-51.

Dibble, C. C. and Manning, B. D. (2013) 'Signal integration by mTORC1 coordinates nutrient input with biosynthetic output', *Nat Cell Biol,* 15(6), pp. 555-64.

Ding, Z., Wu, C. J., Chu, G. C., Xiao, Y., Ho, D., Zhang, J., Perry, S. R., Labrot, E. S., Wu, X., Lis, R., Hoshida, Y., Hiller, D., Hu, B., Jiang, S., Zheng, H., Stegh, A. H., Scott, K. L., Signoretti, S., Bardeesy, N., Wang, Y. A., Hill, D. E., Golub, T. R., Stampfer, M. J., Wong, W. H., Loda, M., Mucci, L., Chin, L. and DePinho, R. A. (2011) 'SMAD4–dependent barrier constrains prostate cancer growth and metastatic progression', *Nature,* 470(7333), pp. 269-273.

Donjacour, A. A. and Cunha, G. R. (1993) 'Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice', *Endocrinology,* 132(6), pp. 2342-50.

Dudley, A. C., Khan, Z. A., Shih, S.-C., Kang, S.-Y., Zwaans, B. M. M., Bischoff, J. and Klagsbrun, M. (2008) 'Calcification of multi-potent, prostate tumor endothelium', *Cancer cell,* 14(3), pp. 201-211.

Edlind, M. P. and Hsieh, A. C. (2014) 'PI3K-AKT-mTOR signaling in prostate cancer progression and androgen deprivation therapy resistance', *Asian J Androl,* 16(3), pp. 378-86.

Emanu, J. C., Avildsen, I. K. and Nelson, C. J. (2016) 'Erectile Dysfunction after Radical Prostatectomy: Prevalence, Medical Treatments, and Psychosocial Interventions', *Current opinion in supportive and palliative care,* 10(1), pp. 102-107.

Etzioni, R., Penson, D. F., Legler, J. M., di Tommaso, D., Boer, R., Gann, P. H. and Feuer, E. J. (2002) 'Overdiagnosis due to prostate-specific antigen screening: lessons from U.S. prostate cancer incidence trends', *J Natl Cancer Inst,* 94(13), pp. 981-90.

Fan, S. J., Snell, C., Turley, H., Li, J. L., McCormick, R., Perera, S. M. W., Heublein, S., Kazi, S., Azad, A., Wilson, C., Harris, A. L. and Goberdhan, D. C. I. (2015) 'PAT4 levels control amino-acid sensitivity of rapamycin-resistant mTORC1 from the Golgi and affect clinical outcome in colorectal cancer', *Oncogene,* 35, pp. 3004.

Feeley, B. T., Krenek, L., Liu, N., Hsu, W. K., Gamradt, S. C., Schwarz, E. M., Huard, J. and Lieberman, J. R. (2006) 'Overexpression of noggin inhibits BMP-mediated growth of osteolytic prostate cancer lesions', *Bone,* 38(2), pp. 154-166.

Feldman, M. E., Apsel, B., Uotila, A., Loewith, R., Knight, Z. A., Ruggero, D. and Shokat, K. M. (2009) 'Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2', *PLoS Biol,* 7(2), pp. e38.

Feletto, E., Bang, A., Cole-Clark, D., Chalasani, V., Rasiah, K. and Smith, D. P. (2015) 'An examination of prostate cancer trends in Australia, England, Canada and USA: Is the Australian death rate too high?', *World J Urol,* 33(11), pp. 1677-87.

Fendt, S.M., Bell, E.L., Keibler, M.A., Davidson, S.M., Wirth, G.J., Fiske, B., Mayers, J.R., Schwab, M., Bellinger, G., Csibi, A. and Patnaik, A. (2013). 'Metformin decreases glucose oxidation and increases the dependency of prostate cancer cells on reductive glutamine metabolism', *Cancer research*, 73(14), pp. 4429–4438.

Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J. W., Comber, H., Forman, D. and Bray, F. (2013) 'Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012', *Eur J Cancer,* 49(6), pp. 1374-403.

Ferraldeschi, R., Welti, J., Luo, J., Attard, G. and de Bono, J. S. (2015) 'Targeting the androgen receptor pathway in castration-resistant prostate cancer: progresses and prospects', *Oncogene,* 34(14), pp. 1745-57.

Ferrís-i-Tortajada, J., Berbel-Tornero, O., Garcia-i-Castell, J., López-Andreu, J. A., Sobrino-Najul, E. and Ortega-García, J. A. (2011) 'Non-dietary environmental risk factors in prostate cancer', *Actas urologicas espanolas,* 35(5), pp. 289-295.

Fisher, J. L., Schmitt, J. F., Howard, M. L., Mackie, P. S., Choong, P. F. and Risbridger, G. P. (2002) 'An in vivo model of prostate carcinoma growth and invasion in bone', *Cell Tissue Res,* 307(3), pp. 337-45.

Freedland, S. J., Humphreys, E. B., Mangold, L. A., Eisenberger, M., Dorey, F. J., Walsh, P. C. and Partin, A. W. (2005) 'Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy', *Jama,* 294(4), pp. 433-9.

Gámez-Valero, A., Monguió-Tortajada, M., Carreras-Planella, L., Franquesa, M. l., Beyer, K. and Borràs, F. E. (2016) 'Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents', *Scientific Reports,* 6, pp. 33641.

Gan, X., Wang, J., Wang, C., Sommer, E., Kozasa, T., Srinivasula, S., Alessi, D., Offermanns, S., Simon, M. I. and Wu, D. (2012) 'PRR5L degradation promotes MTORC2-mediated PKCδ phosphorylation and cell migration downstream of Gα12', *Nature cell biology,* 14(7), pp. 686-696.

Gao, H., Chakraborty, G., Lee-Lim, A. P., Mo, Q., Decker, M., Vonica, A., Shen, R., Brogi, E., Brivanlou, A. H. and Giancotti, F. G. (2012) 'The BMP inhibitor Coco reactivates breast cancer cells at lung metastatic sites', *Cell,* 150(4), pp. 764-79.

Gao, S., Gao, Y., He, Housheng H., Han, D., Han, W., Avery, A., Macoska, Jill A., Liu, X., Chen, S., Ma, F., Chen, S., Balk, Steven P. and Cai, C. (2016) 'Androgen Receptor Tumor Suppressor Function Is Mediated by Recruitment of Retinoblastoma Protein', *Cell Reports,* 17(4), pp. 966-976.

Garisto, J. D. and Klotz, L. (2017) 'Active Surveillance for Prostate Cancer: How to Do It Right', *Oncology (Williston Park),* 31(5), pp. 333-40, 345.

Geller, J., Albert, J. and Yen, S. S. C. (1978) 'Treatment of advanced cancer of prostate with megestrol acetate', *Urology,* 12(5), pp. 537-541.

Gleason, D. F. (1992) 'Histologic grading of prostate cancer: a perspective', *Hum Pathol,* 23(3), pp. 273-9.

Goberdhan, Deborah C., Wilson, C. and Harris, Adrian L. (2016) 'Amino Acid Sensing by mTORC1: Intracellular Transporters Mark the Spot', *Cell Metabolism,* 23(4), pp. 580-589.

Goberdhan, D. C. I. (2010) 'Intracellular amino acid sensing and mTORC1-regulated growth: New ways to block an old target?', *Current opinion in investigational drugs (London, England : 2000),* 11(12), pp. 1360-1367.

Goberdhan, D. C. I., Meredith, D., Boyd, C. A. R. and Wilson, C. (2005) 'PAT-related amino acid transporters regulate growth via a novel mechanism that does not require bulk transport of amino acids', *Development,* 132(10), pp. 2365-2375.

Godoy, A. S., Sotomayor, P. C., Villagran, M., Yacoub, R., Montecinos, V. P., McNerney, E. M., Moser, M., Foster, B. A. and Onate, S. A. (2012) 'Altered corepressor SMRT expression and recruitment to target genes as a mechanism that change the response to androgens in prostate cancer progression', *Biochem Biophys Res Commun,* 423(3), pp. 564-70.

Gottlieb, B., Beitel, L. K., Nadarajah, A., Paliouras, M. and Trifiro, M. (2012) 'The androgen receptor gene mutations database: 2012 update', *Hum Mutat,* 33(5), pp. 887-94.

Gould, S. J. and Raposo, G. (2013) 'As we wait: coping with an imperfect nomenclature for extracellular vesicles', *Journal of Extracellular Vesicles,* 2, pp. 10.3402/jev.v2i0.20389.

Grad, J., Dai, J., Wu, S., Burnstein, K., Grad, J., Dai, J., Wu, S. and Burnstein, K. (1999) 'Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA', *Molecular Endocrinology,* 13, pp. 1896-1911.

Gravis, G., Bladou, F., Salem, N., Macquart-Moulin, G., Serment, G., Camerlo, J., Genre, D., Bardou, V.-J., Maraninchi, D. and Viens, P. (2003) 'Weekly administration of docetaxel for symptomatic metastatic hormone-refractory prostate carcinoma', *Cancer,* 98(8), pp. 1627-1634.

Gregory, C. W., He, B., Johnson, R. T., Ford, O. H., Mohler, J. L., French, F. S. and Wilson, E. M. (2001) 'A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy', *Cancer Res,* 61(11), pp. 4315-9.

Grindstad, T., Andersen, S., Al-Saad, S., Donnem, T., Kiselev, Y., Nordahl Melbø-Jørgensen, C., Skjefstad, K., Busund, L.-T., Bremnes, R. M. and Richardsen, E. (2015) 'High Progesterone Receptor Expression in Prostate Cancer Is Associated with Clinical Failure', *PLOS ONE,* 10(2), pp. e0116691.

Gronberg, H. (2003) 'Prostate cancer epidemiology', *Lancet,* 361(9360), pp. 859-64.
Guertin, D. A. and Sabatini, D. M. (2007) 'Defining the Role of mTOR in Cancer', *Cancer Cell,* 12(1), pp. 9-22.

Guo, Z., Dai, B., Jiang, T., Xu, K., Xie, Y., Kim, O., Nesheiwat, I., Kong, X., Melamed, J., Handratta, V. D., Njar, V. C., Brodie, A. M., Yu, L. R., Veenstra, T. D., Chen, H. and Qiu, Y. (2006) 'Regulation of androgen receptor activity by tyrosine phosphorylation', *Cancer Cell,* 10(4), pp. 309-19.

Guo, Z., Yang, X., Sun, F., Jiang, R., Linn, D. E., Chen, H., Chen, H., Kong, X., Melamed, J., Tepper, C. G., Kung, H. J., Brodie, A. M., Edwards, J. and Qiu, Y. (2009) 'A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth', *Cancer Res,* 69(6), pp. 2305-13.

Haile, S., Lal, A., Myung, J.-K. and Sadar, M. D. (2011) 'FUS/TLS Is a Co-Activator of Androgen Receptor in Prostate Cancer Cells', *PLOS ONE,* 6(9), pp. e24197.

Hamdy, F. C., Autzen, P., Robinson, M. C., Horne, C. H. W., Neal, D. E. and Robson, C. N. (1997) 'Immunolocalization and Messenger RNA Expression of Bone Morphogenetic Protein-6 in Human Benign and Malignant Prostatic Tissue', *Cancer Research,* 57(19), pp. 4427-4431.

Hamdy, F. C., Donovan, J. L., Lane, J. A., Mason, M., Metcalfe, C., Holding, P., Davis, M., Peters, T. J., Turner, E. L., Martin, R. M., Oxley, J., Robinson, M., Staffurth, J., Walsh, E., Bollina, P., Catto, J., Doble, A., Doherty, A., Gillatt, D., Kockelbergh, R., Kynaston, H., Paul, A., Powell, P., Prescott, S., Rosario, D. J., Rowe, E. and Neal, D. E. (2016) '10-Year Outcomes after Monitoring, Surgery, or Radiotherapy for Localized Prostate Cancer', *N Engl J Med,* 375(15), pp. 1415-1424.

Hamoen, E. H., Reukers, D. F., Numans, M. E., Barentsz, J. O., Witjes, J. A. and Rovers, M. M. (2013) 'Discrepancies between guidelines and clinical practice regarding prostate-specific antigen testing', *Fam Pract,* 30(6), pp. 648-54.

Han, J. M., Jeong, S. J., Park, M. C., Kim, G., Kwon, N. H., Kim, H. K., Ha, S. H., Ryu, S. H. and Kim, S. (2012) 'Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway', *Cell,* 149(2), pp. 410-24.

Hankey, B. F., Feuer, E. J., Clegg, L. X., Hayes, R. B., Legler, J. M., Prorok, P. C., Ries, L. A., Merrill, R. M. and Kaplan, R. S. (1999) 'Cancer surveillance series: interpreting trends in prostate cancer--part I: Evidence of the effects of screening in recent prostate cancer incidence, mortality, and survival rates', *J Natl Cancer Inst,* 91(12), pp. 1017-24.

Hanson, P. I. and Cashikar, A. (2012) 'Multivesicular body morphogenesis', *Annu Rev Cell Dev Biol,* 28, pp. 337-62.

Harding, C., Heuser, J. and Stahl, P. (1983) 'Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes', *J Cell Biol,* 97(2), pp. 329-39.

Harris, S. E., Harris, M. A., Mahy, P., Wozney, J., Feng, J. Q. and Mundy, G. R. (1994) 'Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells', *Prostate,* 24(4), pp. 204-11.

Hassanipour-Azgomi, S., Mohammadian-Hafshejani, A., Ghoncheh, M., Towhidi, F., Jamehshorani, S. and Salehiniya, H. (2016) 'Incidence and mortality of prostate cancer and their relationship with the Human Development Index worldwide', *Prostate International,* 4(3), pp. 118-124.

Haudenschild, D. R., Palmer, S. M., Moseley, T. A., You, Z. and Reddi, A. H. (2004) 'Bone Morphogenetic Protein (BMP)-6 Signaling and BMP Antagonist Noggin in Prostate Cancer', *Cancer Research,* 64(22), pp. 8276-8284.

Hay, C. W. and McEwan, I. J. (2012) 'The Impact of Point Mutations in the Human Androgen Receptor: Classification of Mutations on the Basis of Transcriptional Activity', *PLOS ONE,* 7(3), pp. e32514.

Heitman, J., Movva, N. R. and Hall, M. N. (1991) 'Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast', *Science,* 253(5022), pp. 905-9.

Hermanson, O., Glass, C. K. and Rosenfeld, M. G. (2002) 'Nuclear receptor coregulators: multiple modes of modification', *Trends Endocrinol Metab,* 13(2), pp. 55-60.

Heublein, S., Kazi, S., Ogmundsdottir, M. H., Attwood, E. V., Kala, S., Boyd, C. A., Wilson, C. and Goberdhan, D. C. (2010) 'Proton-assisted amino-acid transporters are conserved regulators of proliferation and amino-acid-dependent mTORC1 activation', *Oncogene,* 29(28), pp. 4068-79.

Hill, R., Song, Y., Cardiff, R. D. and Van Dyke, T. (2005) 'Heterogeneous Tumor Evolution Initiated by Loss of pRb Function in a Preclinical Prostate Cancer Model', *Cancer Research,* 65(22), pp. 10243-10254.

Hogan, B. L. (1996) 'Bone morphogenetic proteins in development', *Curr Opin Genet Dev,* 6(4), pp. 432-8.

Horvath, L. G., Henshall, S. M., Kench, J. G., Turner, J. J., Golovsky, D., Brenner, P. C., O'Neill, G. F., Kooner, R., Stricker, P. D., Grygiel, J. J. and Sutherland, R. L. (2004) 'Loss of BMP2, Smad8, and Smad4 expression in prostate cancer progression', *Prostate,* 59(3), pp. 234-42.

Hoshino, A., Costa-Silva, B., Shen, T. L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Molina, H., Kohsaka, S., Di Giannatale, A., Ceder, S., Singh, S., Williams, C., Soplop, N., Uryu, K., Pharmer, L., King, T., Bojmar, L., Davies, A. E., Ararso, Y., Zhang, T., Zhang, H., Hernandez, J., Weiss, J. M., Dumont-Cole, V. D., Kramer, K., Wexler, L. H., Narendran, A., Schwartz, G. K., Healey, J. H., Sandstrom, P., Labori, K. J., Kure, E. H., Grandgenett, P. M., Hollingsworth, M. A., de Sousa, M., Kaur, S., Jain, M., Mallya, K., Batra, S. K., Jarnagin, W. R., Brady, M. S., Fodstad, O., Muller, V., Pantel, K., Minn, A. J., Bissell, M. J., Garcia, B. A., Kang, Y., Rajasekhar, V. K., Ghajar, C. M., Matei, I., Peinado, H., Bromberg, J. and Lyden, D. (2015) 'Tumour exosome integrins determine organotropic metastasis', *Nature,* 527(7578), pp. 329-35.

Hoyland, K., Vasdev, N., Abrof, A. and Boustead, G. (2014) 'Post-Radical Prostatectomy Incontinence: Etiology and Prevention', *Reviews in Urology,* 16(4), pp. 181-188.

Hsieh, A. C., Costa, M., Zollo, O., Davis, C., Feldman, M. E., Testa, J. R., Meyuhas, O., Shokat, K. M. and Ruggero, D. (2010) 'Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E', *Cancer Cell,* 17(3), pp. 249-61.

Hsieh, A. C., Liu, Y., Edlind, M. P., Ingolia, N. T., Janes, M. R., Sher, A., Shi, E. Y., Stumpf, C. R., Christensen, C., Bonham, M. J., Wang, S., Ren, P., Martin, M., Jessen, K., Feldman, M. E., Weissman, J. S., Shokat, K. M., Rommel, C. and Ruggero, D. (2012) 'The translational landscape of mTOR signalling steers cancer initiation and metastasis', *Nature,* 485(7396), pp. 55-61.

Hu, R., Lu, C., Mostaghel, E. A., Yegnasubramanian, S., Gurel, M., Tannahill, C., Edwards, J., Isaacs, W. B., Nelson, P. S., Bluemn, E., Plymate, S. R. and Luo, J. (2012) 'Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer', *Cancer Res,* 72(14), pp. 3457-62.

Huang, J., Dibble, C. C., Matsuzaki, M. and Manning, B. D. (2008) 'The TSC1-TSC2 complex is required for proper activation of mTOR complex 2', *Mol Cell Biol,* 28(12), pp. 4104-15.

Huang, Y., Jiang, X., Liang, X. and Jiang, G. (2018) 'Molecular and cellular mechanisms of castration resistant prostate cancer', *Oncology Letters,* 15(5), pp. 6063-6076.

Huggins, C. and Hodges, C. V. (2002) 'Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941', *J Urol,* 168(1), pp. 9-12.

Huggins, C., Stevens, R. E., Jr and Hodges, C. V. (1941) 'Studies on prostatic cancer: Ii. the effects of castration on advanced carcinoma of the prostate gland', *Archives of Surgery,* 43(2), pp. 209-223.

Huncharek, M., Haddock, K. S., Reid, R. and Kupelnick, B. (2010) 'Smoking as a risk factor for prostate cancer: a meta-analysis of 24 prospective cohort studies', *Am J Public Health,* 100(4), pp. 693-701.

Hurley, J. H. (2010) 'The ESCRT Complexes', *Critical reviews in biochemistry and molecular biology,* 45(6), pp. 463-487.

Ibrahim, T., Flamini, E., Mercatali, L., Sacanna, E., Serra, P. and Amadori, D. (2010) 'Pathogenesis of osteoblastic bone metastases from prostate cancer', *Cancer,* 116(6), pp. 1406-1418.

Ide, H., Yoshida, T., Matsumoto, N., Aoki, K., Osada, Y., Sugimura, T. and Terada, M. (1997) 'Growth Regulation of Human Prostate Cancer Cells by Bone Morphogenetic Protein-2', *Cancer Research,* 57(22), pp. 5022-5027.

Inoki, K., Li, Y., Xu, T. and Guan, K.-L. (2003) 'Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling', *Genes & Development,* 17(15), pp. 1829-1834.

Jacinto, E. and Lorberg, A. (2008) 'TOR regulation of AGC kinases in yeast and mammals', *Biochemical Journal,* 410(1), pp. 19-37.

Jaiswal, J. K., Andrews, N. W. and Simon, S. M. (2002) 'Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells', *The Journal of Cell Biology,* 159(4), pp. 625-635.

Jeppesen, D. K., Hvam, M. L., Primdahl-Bengtson, B., Boysen, A. T., Whitehead, B., Dyrskjot, L., Orntoft, T. F., Howard, K. A. and Ostenfeld, M. S. (2014) 'Comparative analysis of discrete exosome fractions obtained by differential centrifugation', *J Extracell Vesicles,* 3, pp. 25011.

Jewell, J. L., Kim, Y. C., Russell, R. C., Yu, F. X., Park, H. W., Plouffe, S. W., Tagliabracci, V. S. and Guan, K. L. (2015) 'Metabolism. Differential regulation of mTORC1 by leucine and glutamine', *Science,* 347(6218), pp. 194-8.

Jewell, J. L., Russell, R. C. and Guan, K. L. (2013) 'Amino acid signalling upstream of mTOR', *Nat Rev Mol Cell Biol,* 14(3), pp. 133-9.

Jiao, J., Wang, S., Qiao, R., Vivanco, I., Watson, P. A., Sawyers, C. L. and Wu, H. (2007) 'Murine Cell Lines Derived from Pten Null Prostate Cancer Show the Critical Role of PTEN in Hormone Refractory Prostate Cancer Development', *Cancer Research,* 67(13), pp. 6083-6091.

Jin, R., Sterling, J. A., Edwards, J. R., DeGraff, D. J., Lee, C., Park, S. I. and Matusik, R. J. (2013) 'Activation of NF-kappa B Signaling Promotes Growth of Prostate Cancer Cells in Bone', *PLoS ONE,* 8(4), pp. e60983.

Johns, L. E. and Houlston, R. S. (2003) 'A systematic review and meta-analysis of familial prostate cancer risk', *BJU Int,* 91(9), pp. 789-94.

Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L. and Turbide, C. (1987) 'Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)', *J Biol Chem,* 262(19), pp. 9412-20.

Jones, C. M., Lyons, K. M. and Hogan, B. L. (1991) 'Involvement of Bone Morphogenetic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse', *Development,* 111(2), pp. 531-42.

Josson, S., Matsuoka, Y., Chung, L. W. K., Zhau, H. E. and Wang, R. (2010) 'Tumor stroma co-evolution in prostate cancer progression and metastasis', *Seminars in cell & developmental biology,* 21(1), pp. 26-32.

Jozwik, K. M. and Carroll, J. S. (2012) 'Pioneer factors in hormone-dependent cancers', *Nat Rev Cancer,* 12(6), pp. 381-5.

Jung, J., Genau, H. M. and Behrends, C. (2015) 'Amino Acid-Dependent mTORC1 Regulation by the Lysosomal Membrane Protein SLC38A9', *Mol Cell Biol,* 35(14), pp. 2479-94.

Kang, S. A., Pacold, M. E., Cervantes, C. L., Lim, D., Lou, H. J., Ottina, K., Gray, N. S., Turk, B. E., Yaffe, M. B. and Sabatini, D. M. (2013) 'mTORC1 Phosphorylation Sites Encode Their Sensitivity to Starvation and Rapamycin', *Science,* 341(6144).

Karlou, M., Tzelepi, V. and Efstathiou, E. (2010) 'Therapeutic targeting of the prostate cancer microenvironment', *Nature Reviews Urology,* 7, pp. 494.

Karunakaran, S., Ramachandran, S., Coothankandaswamy, V., Elangovan, S., Babu, E., Periyasamy-Thandavan, S., Gurav, A., Gnanaprakasam, J. P., Singh, N., Schoenlein, P. V., Prasad, P. D., Thangaraju, M. and Ganapathy, V. (2011) 'SLC6A14 (ATB0,+) protein, a highly concentrative and broad specific amino acid transporter, is a novel and effective drug target for treatment of estrogen receptor-positive breast cancer', *J Biol Chem,* 286(36), pp. 31830-8.

Kasperzyk, J. L., Shappley, W. V., III, Kenfield, S. A., Mucci, L. A., Kurth, T., Ma, J., Stampfer, M. J. and Sanda, M. G. (2011) 'Watchful Waiting and Quality of Life Among Prostate Cancer Survivors in the Physicians' Health Study', *The Journal of Urology,* 186(5), pp. 1862-1867.

Keerthikumar, S., Gangoda, L., Liem, M., Fonseka, P., Atukorala, I., Ozcitti, C. and Mathivanan, S. (2015). 'Proteogenomic analysis reveals exosomes are more oncogenic than ectosomes', *Oncotarget*, *6*(17), pp. 15375.

Keith, C. T. and Schreiber, S. L. (1995) 'PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints', *Science,* 270(5233), pp. 50-1.

Kelly, S. P., Anderson, W. F., Rosenberg, P. S. and Cook, M. B. (2018) 'Past, Current, and Future Incidence Rates and Burden of Metastatic Prostate Cancer in the United States', *Eur Urol Focus,* 4(1), pp. 121-127.

Khan, M. A. and Partin, A. W. (2002) 'Evolving Role of Pro-PSA as a New Serum Marker for the Early Detection of Prostate Cancer', *Rev Urol,* 4(4), pp. 198-200.

Kim, I. Y., Lee, D.-H., Ahn, H.-J., Tokunaga, H., Song, W., Devereaux, L. M., Jin, D., Sampath, T. K. and Morton, R. A. (2000) 'Expression of Bone Morphogenetic Protein Receptors Type-IA, -IB, and -II Correlates with Tumor Grade in Human Prostate Cancer Tissues', *Cancer Research,* 60(11), pp. 2840-2844.

Kim, I. Y., Lee, D.-H., Lee, D. K., Ahn, H.-J., Kim, M. M., Kim, S. J. and Morton, R. A. (2004) 'Loss of expression of bone morphogenetic protein receptor type II in human prostate cancer cells', *Oncogene,* 23, pp. 7651.

Kim, S., Kim, D. H., Jung, W. H. and Koo, J. S. (2013) 'Metabolic phenotypes in triple-negative breast cancer', *Tumour Biol,* 34(3), pp. 1699-712.

Kim, Y. J., Cho, K. H., Pyo, H. R., Lee, K. H., Moon, S. H., Kim, T. H., Shin, K. H., Kim, J. Y., Kim, Y. K. and Lee, S. B. (2015) 'Radical prostatectomy versus external beam radiotherapy for localized prostate cancer: Comparison of treatment outcomes', *Strahlenther Onkol,* 191(4), pp. 321-9.

Kinkade, C. W., Castillo-Martin, M., Puzio-Kuter, A., Yan, J., Foster, T. H., Gao, H., Sun, Y., Ouyang, X., Gerald, W. L., Cordon-Cardo, C. and Abate-Shen, C. (2008) 'Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model', *The Journal of Clinical Investigation,* 118(9), pp. 3051-3064.

Knuuttila, M., Yatkin, E., Kallio, J., Savolainen, S., Laajala, T. D., Aittokallio, T., Oksala, R., Häkkinen, M., Keski-Rahkonen, P., Auriola, S., Poutanen, M. and Mäkelä, S. (2014) 'Castration Induces Up-Regulation of Intratumoral Androgen Biosynthesis and Androgen Receptor Expression in an Orthotopic VCaP Human Prostate Cancer Xenograft Model', *The American Journal of Pathology,* 184(8), pp. 2163-2173.

Kobayashi, A., Okuda, H., Xing, F., Pandey, P. R., Watabe, M., Hirota, S., Pai, S. K., Liu, W., Fukuda, K., Chambers, C., Wilber, A. and Watabe, K. (2011) 'Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone', *J Exp Med,* 208(13), pp. 2641-55.

Koivisto, P., Kolmer, M., Visakorpi, T. and Kallioniemi, O. P. (1998) 'Androgen receptor gene and hormonal therapy failure of prostate cancer', *Am J Pathol,* 152(1), pp. 1-9.

Kooijman, E. E., Chupin, V., de Kruijff, B. and Burger, K. N. (2003) 'Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid', *Traffic,* 4(3), pp. 162-74.

Kowal, P., Goodkind, D. and He, W. (2016) 'An Ageing Word: 2015', Available: U.S. Census Bureau.

Kremer, C. L., Klein, R. R., Mendelson, J., Browne, W., Samadzedeh, L. K., Vanpatten, K., Highstrom, L., Pestano, G. A. and Nagle, R. B. (2006) 'Expression of mTOR signaling pathway markers in prostate cancer progression', *Prostate,* 66(11), pp. 1203-12.

Krušlin, B., Ulamec, M. and Tomas, D. (2015) 'Prostate cancer stroma: an important factor in cancer growth and progression', *Bosnian Journal of Basic Medical Sciences,* 15(2), pp. 1-8.

Kucharzewska, P., & Belting, M. (2013). 'Emerging roles of extracellular vesicles in the adaptive response of tumour cells to microenvironmental stress', *Journal of Extracellular Vesicles*, *2*(10), pp. 3402/jev.v2i0.20304.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N. R. and Hall, M. N. (1993) 'Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression', *Cell,* 73(3), pp. 585-96.

Kvåle, R., Auvinen, A., Adami, H.-O., Klint, Å., Hernes, E., Møller, B., Pukkala, E., Storm, H. H., Tryggvadottir, L., Tretli, S., Wahlqvist, R., Weiderpass, E. and Bray, F. (2007) 'Interpreting Trends in Prostate Cancer Incidence and Mortality in the Five Nordic Countries', *JNCI: Journal of the National Cancer Institute,* 99(24), pp. 1881-1887.

Lamm, M. L., Podlasek, C. A., Barnett, D. H., Lee, J., Clemens, J. Q., Hebner, C. M. and Bushman, W. (2001) 'Mesenchymal factor bone morphogenetic protein 4 restricts ductal budding and branching morphogenesis in the developing prostate', *Dev Biol,* 232(2), pp. 301-14.

Lamont, K. R. and Tindall, D. J. (2011) 'Minireview: Alternative activation pathways for the androgen receptor in prostate cancer', *Mol Endocrinol,* 25(6), pp. 897-907.

Lao, L., Franklin, R. B. and Costello, L. C. (1993) 'High-affinity L-aspartate transporter in prostate epithelial cells that is regulated by testosterone', *Prostate,* 22(1), pp. 53-63.

Laplante, M. and Sabatini, D. M. (2009) 'mTOR signaling at a glance', *J Cell Sci,* 122(Pt 20), pp. 3589-94.

Larranaga, N., Galceran, J., Ardanaz, E., Franch, P., Navarro, C., Sanchez, M. J. and Pastor-Barriuso, R. (2010) 'Prostate cancer incidence trends in Spain before and during the prostate-specific antigen era: impact on mortality', *Ann Oncol,* 21 Suppl 3, pp. iii83-89.

Lee, C., Sutkowski, D. M., Sensibar, J. A., Zelner, D., Kim, I., Amsel, I., Shaw, N., Prins, G. S. and Kozlowski, J. M. (1995) 'Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone', *Endocrinology,* 136(2), pp. 796-803.

Lee, G. T., Jung, Y. S., Ha, Y. S., Kim, J. H., Kim, W. J. and Kim, I. Y. (2013) 'Bone morphogenetic protein-6 induces castration resistance in prostate cancer cells through tumor infiltrating macrophages', *Cancer Sci,* 104(8), pp. 1027-32.

Lee, G. T., Kang, D. I., Ha, Y. S., Jung, Y. S., Chung, J., Min, K., Kim, T. H., Moon, K. H., Chung, J. M., Lee, D. H., Kim, W. J. and Kim, I. Y. (2014) 'Prostate cancer bone metastases acquire resistance to androgen deprivation via WNT5A-mediated BMP-6 induction', *British Journal of Cancer,* 110(6), pp. 1634-1644.

Leiblich, A., Marsden, L., Gandy, C., Corrigan, L., Jenkins, R., Hamdy, F. and Wilson, C. (2012) 'Bone morphogenetic protein- and mating-dependent secretory cell growth and migration in the Drosophila accessory gland', *Proceedings of the National Academy of Sciences of the United States of America,* 109(47), pp. 19292-19297.

Leitzmann, M. F. and Rohrmann, S. (2012) 'Risk factors for the onset of prostatic cancer: age, location, and behavioral correlates', *Clin Epidemiol,* 4, pp. 1-11.

Lin, D., Gout, P. W. and Wang, Y. (2013) 'Lessons from in-vivo models of castration-resistant prostate cancer', *Curr Opin Urol,* 23(3), pp. 214-9.

Lin, M. F., Meng, T. C., Rao, P. S., Chang, C., Schonthal, A. H. and Lin, F. F. (1998) 'Expression of human prostatic acid phosphatase correlates with androgen-stimulated cell proliferation in prostate cancer cell lines', *J Biol Chem,* 273(10), pp. 5939-47.

Locke, J. A., Guns, E. S., Lubik, A. A., Adomat, H. H., Hendy, S. C., Wood, C. A., Ettinger, S. L., Gleave, M. E. and Nelson, C. C. (2008) 'Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer', *Cancer Res,* 68(15), pp. 6407-15.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M. N. (2002) 'Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control', *Mol Cell,* 10(3), pp. 457-68.

Lonergan, P. E. and Tindall, D. J. (2011) 'Androgen receptor signaling in prostate cancer development and progression', *J Carcinog,* 10, pp. 20.

Lundholm, M., Schröder, M., Nagaeva, O., Baranov, V., Widmark, A., Mincheva-Nilsson, L. and Wikström, P. (2014) 'Prostate Tumor-Derived Exosomes Down-Regulate NKG2D Expression on Natural Killer Cells and CD8+ T Cells: Mechanism of Immune Evasion', *PLOS ONE,* 9(9), pp. e108925.

Lyons, K. M., Pelton, R. W. and Hogan, B. L. (1989) 'Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-beta-like genes coordinately regulate aspects of embryonic development', *Genes Dev,* 3(11), pp. 1657-68.

Maas, S. L. N., Breakefield, X. O. and Weaver, A. M. (2017) 'Extracellular Vesicles: Unique Intercellular Delivery Vehicles', *Trends in Cell Biology,* 27(3), pp. 172-188.

MacLean, H. E., Chu, S., Warne, G. L. and Zajac, J. D. (1993) 'Related individuals with different androgen receptor gene deletions', *Journal of Clinical Investigation,* 91(3), pp. 1123-1128.

Mahajan, N. P., Liu, Y., Majumder, S., Warren, M. R., Parker, C. E., Mohler, J. L., Earp, H. S. and Whang, Y. E. (2007) 'Activated Cdc42-associated kinase Ack1 promotes prostate cancer progression via androgen receptor tyrosine phosphorylation', *Proceedings of the National Academy of Sciences of the United States of America,* 104(20), pp. 8438-8443.

Majumder, P. K., Yeh, J. J., George, D. J., Febbo, P. G., Kum, J., Xue, Q., Bikoff, R., Ma, H., Kantoff, P. W., Golub, T. R., Loda, M. and Sellers, W. R. (2003) 'Prostate intraepithelial neoplasia induced by prostate restricted Akt activation: The MPAKT model', *Proceedings of the National Academy of Sciences,* 100(13), pp. 7841-7846.

Manning, B. D. and Cantley, L. C. (2003) 'United at last: the tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signalling', *Biochem Soc Trans,* 31(Pt 3), pp. 573-8.

Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J. and Cantley, L. C. (2002) 'Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway', *Mol Cell,* 10(1), pp. 151-62.

Masuda, H., Fukabori, Y., Nakano, K., Shimizu, N. and Yamanaka, H. (2004) 'Expression of bone morphogenetic protein-7 (BMP-7) in human prostate', *Prostate,* 59(1), pp. 101-6.

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Faure, J., Blanc, N. S., Matile, S., Dubochet, J., Sadoul, R., Parton, R. G., Vilbois, F. and Gruenberg, J. (2004) 'Role of LBPA and Alix in multivesicular liposome formation and endosome organization', *Science,* 303(5657), pp. 531-4.

Maxfield, F. R. and McGraw, T. E. (2004) 'Endocytic recycling', *Nat Rev Mol Cell Biol,* 5(2), pp. 121-32.

Mayor, S. (2017) 'PSA screening significantly cuts risk of death from prostate cancer, review finds', *BMJ,* 358.

Melo, S. A., Luecke, L. B., Kahlert, C., Fernandez, A. F., Gammon, S. T., Kaye, J., LeBleu, V. S., Mittendorf, E. A., Weitz, J., Rahbari, N., Reissfelder, C., Pilarsky, C., Fraga, M. F., Piwnica-Worms, D. and Kalluri, R. (2015) 'Glypican-1 identifies cancer exosomes and detects early pancreatic cancer', *Nature,* 523(7559), pp. 177-82.

Metcalfe, C., Evans, S., Ibrahim, F., Patel, B., Anson, K., Chinegwundoh, F., Corbishley, C., Gillatt, D., Kirby, R., Muir, G., Nargund, V., Popert, R., Persad, R. and Ben-Shlomo, Y. (2008) 'Pathways to diagnosis for Black men and White men found to have prostate cancer: the PROCESS cohort study', *Br J Cancer,* 99(7), pp. 1040-5.

Miyazaki, H., Watabe, T., Kitamura, T. and Miyazono, K. (2004) 'BMP signals inhibit proliferation and in vivo tumor growth of androgen-insensitive prostate carcinoma cells', *Oncogene,* 23, pp. 9326.

Miyazono, K., Kamiya, Y. and Morikawa, M. (2010) 'Bone morphogenetic protein receptors and signal transduction', *J Biochem,* 147(1), pp. 35-51.

Mizutani, K., Terazawa, R., Kameyama, K., Kato, T., Horie, K., Tsuchiya, T., Seike, K., Ehara, H., Fujita, Y., Kawakami, K., Ito, M. and Deguchi, T. (2014) 'Isolation of prostate cancer-related exosomes', *Anticancer Res,* 34(7), pp. 3419-23.

Mobius, W., Ohno-Iwashita, Y., van Donselaar, E. G., Oorschot, V. M., Shimada, Y., Fujimoto, T., Heijnen, H. F., Geuze, H. J. and Slot, J. W. (2002) 'Immunoelectron microscopic localization of cholesterol using biotinylated and non-cytolytic perfringolysin O', *J Histochem Cytochem,* 50(1), pp. 43-55.

Mol, E. A., Goumans, M. J., Doevendans, P. A., Sluijter, J. P. G. and Vader, P. (2017) 'Higher functionality of extracellular vesicles isolated using size-exclusion chromatography compared to ultracentrifugation', *Nanomedicine,* 13(6), pp. 2061-2065.

Momen-Heravi, F., Balaj, L., Alian, S., Mantel, P. Y., Halleck, A. E., Trachtenberg, A. J., Soria, C. E., Oquin, S., Bonebreak, C. M., Saracoglu, E., Skog, J. and Kuo, W. P. (2013) 'Current methods for the isolation of extracellular vesicles', *Biol Chem,* 394(10), pp. 1253-62.

Montaner-Tarbes, S., Borrás, F. E., Montoya, M., Fraile, L. and del Portillo, H. A. (2016) 'Serum-derived exosomes from non-viremic animals previously exposed to the porcine respiratory and reproductive virus contain antigenic viral proteins', *Veterinary Research,* 47(1), pp. 59.

Montgomery, R. B., Mostaghel, E. A., Vessella, R., Hess, D. L., Kalhorn, T. F., Higano, C. S., True, L. D. and Nelson, P. S. (2008) 'Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth', *Cancer Res,* 68(11), pp. 4447-54.

Morgan, T. M., Lange, P. H., Porter, M. P., Lin, D. W., Ellis, W. J., Gallaher, I. S. and Vessella, R. L. (2009) 'Disseminated tumor cells in prostate cancer patients after radical prostatectomy and without evidence of disease predicts biochemical recurrence', *Clin Cancer Res,* 15(2), pp. 677-83.

Morrissey, C., Brown, L. G., Pitts, T. E., Vessella, R. L. and Corey, E. (2010) 'Bone morphogenetic protein 7 is expressed in prostate cancer metastases and its effects on prostate tumor cells depend on cell phenotype and the tumor microenvironment', *Neoplasia,* 12(2), pp. 192-205.

Mostaghel, E. A., Page, S. T., Lin, D. W., Fazli, L., Coleman, I. M., True, L. D., Knudsen, B., Hess, D. L., Nelson, C. C., Matsumoto, A. M., Bremner, W. J., Gleave, M. E. and Nelson, P. S. (2007) 'Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer', *Cancer Res,* 67(10), pp. 5033-41.

Mulholland, D. J., Tran, L. M., Li, Y., Cai, H., Morim, A., Wang, S., Plaisier, S., Garraway, I. P., Huang, J., Graeber, T. G. and Wu, H. (2011) 'Cell Autonomous Role of PTEN in Regulating Castration-Resistant Prostate Cancer Growth', *Cancer cell,* 19(6), pp. 792-804.

Muhsin-Sharafaldine, M. R., Saunderson, S. C., Dunn, A. C., Faed, J. M., Kleffmann, T. and McLellan, A. D. (2016). 'Procoagulant and immunogenic properties of melanoma exosomes, microvesicles and apoptotic vesicles', *Oncotarget*, *7*(35), pp. 56279.

Nadiminty, N., Tummala, R., Liu, C., Yang, J., Lou, W., Evans, C. P. and Gao, A. C. (2013) 'NF-κB2/p52 Induces Resistance to Enzalutamide in Prostate Cancer: Role of Androgen Receptor and Its Variants', *Molecular cancer therapeutics,* 12(8), pp. 1629-1637.

Naito, Y., Yoshioka, Y., Yamamoto, Y. and Ochiya, T. (2017) 'How cancer cells dictate their microenvironment: present roles of extracellular vesicles', *Cellular and Molecular Life Sciences,* 74(4), pp. 697-713.

Nakabayashi, M., Werner, L., Courtney, K. D., Buckle, G., Oh, W. K., Bubley, G. J., Hayes, J. H., Weckstein, D., Elfiky, A., Sims, D. M., Kantoff, P. W. and Taplin, M. E. (2012) 'Phase II trial of RAD001 and bicalutamide for castration-resistant prostate cancer', *BJU Int,* 110(11), pp. 1729-35.

Nakazawa, M., Antonarakis, E. S. and Luo, J. (2014) 'Androgen receptor splice variants in the era of enzalutamide and abiraterone', *Horm Cancer,* 5(5), pp. 265-73.

Nantermet, P. V., Xu, J., Yu, Y., Hodor, P., Holder, D., Adamski, S., Gentile, M. A., Kimmel, D. B., Harada, S., Gerhold, D., Freedman, L. P. and Ray, W. J. (2004) 'Identification of genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland', *J Biol Chem,* 279(2), pp. 1310-22.

Nardella, C., Carracedo, A., Alimonti, A., Hobbs, R. M., Clohessy, J. G., Chen, Z., Egia, A., Fornari, A., Fiorentino, M., Loda, M., Kozma, S. C., Thomas, G., Cordon-Cardo, C. and Pandolfi, P. P. (2009) 'Differential requirement of mTOR in post-mitotic tissues and tumorigenesis', *Science signaling,* 2(55), pp. ra2-ra2.

Nelson, P. S., Clegg, N., Arnold, H., Ferguson, C., Bonham, M., White, J., Hood, L. and Lin, B. (2002) 'The program of androgen-responsive genes in neoplastic prostate epithelium', *Proceedings of the National Academy of Sciences of the United States of America,* 99(18), pp. 11890-11895.

Nickerson, D. P., West, M., Henry, R. and Odorizzi, G. (2010) 'Regulators of Vps4 ATPase Activity at Endosomes Differentially Influence the Size and Rate of Formation of Intralumenal Vesicles', *Molecular Biology of the Cell,* 21(6), pp. 1023-1032.

Nicklin, P., Bergman, P., Zhang, B., Triantafellow, E., Wang, H., Nyfeler, B., Yang, H., Hild, M., Kung, C., Wilson, C., Myer, V. E., MacKeigan, J. P., Porter, J. A., Wang, Y. K., Cantley, L. C., Finan, P. M. and Murphy, L. O. (2009) 'Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy', *Cell,* 136(3), pp. 521-534.

Nordin, J. Z., Lee, Y., Vader, P., Mager, I., Johansson, H. J., Heusermann, W., Wiklander, O. P., Hallbrink, M., Seow, Y., Bultema, J. J., Gilthorpe, J., Davies, T., Fairchild, P. J., Gabrielsson, S., Meisner-Kober, N. C., Lehtio, J., Smith, C. I., Wood, M. J. and El Andaloussi, S. (2015) 'Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties', *Nanomedicine,* 11(4), pp. 879-83.

Odorizzi, G., Babst, M. and Emr, S. D. (1998) 'Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body', *Cell,* 95(6), pp. 847-58.

Ögmundsdóttir, M. H., Heublein, S., Kazi, S., Reynolds, B., Visvalingam, S. M., Shaw, M. K. and Goberdhan, D. C. I. (2012) 'Proton-Assisted Amino Acid Transporter PAT1 Complexes with Rag GTPases and Activates TORC1 on Late Endosomal and Lysosomal Membranes', *PLOS ONE,* 7(5), pp. e36616.

Pan, B. T., Teng, K., Wu, C., Adam, M. and Johnstone, R. M. (1985) 'Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes', *J Cell Biol,* 101(3), pp. 942-8.

Pan, J., Ding, M., Xu, K., Yang, C., and Mao, L. J. (2017) 'Exosomes in diagnosis and therapy of prostate cancer', *Oncotarget*, 8(57), pp. 97693-97700.

Paralkar, V. M., Vail, A. L., Grasser, W. A., Brown, T. A., Xu, H., Vukicevic, S., Ke, H. Z., Qi, H., Owen, T. A. and Thompson, D. D. (1998) 'Cloning and characterization of a novel member of the transforming growth factor-beta/bone morphogenetic protein family', *J Biol Chem,* 273(22), pp. 13760-7.

Pardali, K., Kowanetz, M., Heldin, C. H. and Moustakas, A. (2005) 'Smad pathway-specific transcriptional regulation of the cell cycle inhibitor p21(WAF1/Cip1)', *J Cell Physiol,* 204(1), pp. 260-72.

Parmigiani, A., Nourbakhsh, A., Ding, B., Wang, W., Kim, Y. C., Akopiants, K., Guan, K.-L., Karin, M. and Budanov, A. V. (2014) 'Sestrins Inhibit mTORC1 Kinase Activation Through the GATOR Complex', *Cell reports,* 9(4), pp. 1281-1291.

Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Coscia, C., Iessi, E., Logozzi, M.A., Molinari, A. and Colone, M. (2009) 'Microenvironmental pH is a key factor for exosome traffic in tumor cells'. *Journal of Biological Chemistry*, pp. jbc-M109.

Patel, A. R. and Klein, E. A. (2009) 'Risk factors for prostate cancer', *Nat Clin Pract Urol,* 6(2), pp. 87-95.

Peak, T. C., Kasica, N. P., Panigrahi, G. K., Patterson, S. L., Singh, R., Hemal, A. K., Bitting, R. L. and Deep, G. (2017) 'Abstract 3187: Role of exosome secretion in the survival of

enzalutamide-resistant prostate cancer cells: Syntaxin 6 as a novel therapeutic target', *Cancer Research,* 77(13 Supplement), pp. 3187-3187.

Perez-Hernandez, D., Gutierrez-Vazquez, C., Jorge, I., Lopez-Martin, S., Ursa, A., Sanchez-Madrid, F., Vazquez, J. and Yanez-Mo, M. (2013) 'The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes', *J Biol Chem,* 288(17), pp. 11649-61.

Petit, C. S., Roczniak-Ferguson, A. and Ferguson, S. M. (2013) 'Recruitment of folliculin to lysosomes supports the amino acid–dependent activation of Rag GTPases', *The Journal of Cell Biology,* 202(7), pp. 1107-1122.

Pezaro, C., Woo, H. H. and Davis, I. D. (2014) 'Prostate cancer: measuring PSA', *Internal Medicine Journal,* 44(5), pp. 433-440.

Pham, L. K., Liang, M., Adisetiyo, H. A., Liao, C.-P., Cohen, M. B., Tahara, S. M., Frenkel, B., Kasahara, N. and Roy-Burman, P. (2013) 'Contextual Effect of Repression of Bone Morphogenetic Protein Activity in Prostate Cancer', *Endocrine-related cancer,* 20(6), pp. 861-874.

Pischon, T., Boeing, H., Weikert, S., Allen, N., Key, T., Johnsen, N. F., Tjonneland, A., Severinsen, M. T., Overvad, K., Rohrmann, S., Kaaks, R., Trichopoulou, A., Zoi, G., Trichopoulos, D., Pala, V., Palli, D., Tumino, R., Sacerdote, C., Bueno-de-Mesquita, H. B., May, A., Manjer, J., Wallstrom, P., Stattin, P., Hallmans, G., Buckland, G., Larranaga, N., Chirlaque, M. D., Martinez, C., Redondo Cornejo, M. L., Ardanaz, E., Bingham, S., Khaw, K. T., Rinaldi, S., Slimani, N., Jenab, M. and Riboli, E. (2008) 'Body size and risk of prostate cancer in the European prospective investigation into cancer and nutrition', *Cancer Epidemiol Biomarkers Prev,* 17(11), pp. 3252-61.

Qiu, T., Grizzle, W. E., Oelschlager, D. K., Shen, X. and Cao, X. (2007) 'Control of prostate cell growth: BMP antagonizes androgen mitogenic activity with incorporation of MAPK signals in Smad1', *The EMBO Journal,* 26(2), pp. 346-357.

Qu, Y., Dai, B., Ye, D., Kong, Y., Chang, K., Jia, Z., Yang, X., Zhang, H., Zhu, Y. and Shi, G. (2015) 'Constitutively Active AR-V7 Plays an Essential Role in the Development and Progression of Castration-Resistant Prostate Cancer', *Scientific Reports,* 5, pp. 7654.
Rahman, M. S., Akhtar, N., Jamil, H. M., Banik, R. S. and Asaduzzaman, S. M. (2015) 'TGF-β/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation', *Bone Research,* 3, pp. 15005.

Rai, J. S., Henley, M. J. and Ratan, H. L. (2010) 'Mammalian target of rapamycin: A new target in prostate cancer', *Urologic Oncology: Seminars and Original Investigations,* 28(2), pp. 134-138.

Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J. and Geuze, H. J. (1996) 'B lymphocytes secrete antigen-presenting vesicles', *The Journal of Experimental Medicine,* 183(3), pp. 1161-1172.

Raposo, G. and Stoorvogel, W. (2013) 'Extracellular vesicles: Exosomes, microvesicles, and friends', *The Journal of Cell Biology,* 200(4), pp. 373-383.

Read, J., Ingram, A., Al Saleh, H. A., Platko, K., Gabriel, K., Kapoor, A., Pinthus, J., Majeed, F., Qureshi, T. and Al-Nedawi, K. (2017) 'Nuclear transportation of exogenous epidermal growth factor receptor and androgen receptor via extracellular vesicles', *European Journal of Cancer,* 70, pp. 62-74.

Rebsamen, M., Pochini, L., Stasyk, T., de Araujo, M. E., Galluccio, M., Kandasamy, R. K., Snijder, B., Fauster, A., Rudashevskaya, E. L., Bruckner, M., Scorzoni, S., Filipek, P. A., Huber, K. V., Bigenzahn, J. W., Heinz, L. X., Kraft, C., Bennett, K. L., Indiveri, C., Huber, L. A. and Superti-Furga, G. (2015) 'SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1', *Nature,* 519(7544), pp. 477-81.

Reddi, A. H. (1998) 'Cartilage-derived morphogenetic proteins and cartilage morphogenesis', *Microscopy Research and Technique,* 43(2), pp. 131-136.

Resnick, M. J., Koyama, T., Fan, K. H., Albertsen, P. C., Goodman, M., Hamilton, A. S., FHoffman, R. M., Potosky, A. L., Stanford, J. L., Stroup, A. M., Van Horn, R. L. and Penson, D. F. (2013) 'Long-term functional outcomes after treatment for localized prostate cancer', *N Engl J Med,* 368(5), pp. 436-45.

Robinson, D., Van Allen, E. M., Wu, Y. M., Schultz, N., Lonigro, R. J., Mosquera, J. M., Montgomery, B., Taplin, M. E., Pritchard, C. C., Attard, G., Beltran, H., Abida, W., Bradley, R. K., Vinson, J., Cao, X., Vats, P., Kunju, L. P., Hussain, M., Feng, F. Y., Tomlins, S. A., Cooney, K. A., Smith, D. C., Brennan, C., Siddiqui, J., Mehra, R., Chen, Y., Rathkopf, D. E., Morris, M. J., Solomon, S. B., Durack, J. C., Reuter, V. E., Gopalan, A., Gao, J., Loda, M., Lis, R. T., Bowden, M., Balk, S. P., Gaviola, G., Sougnez, C., Gupta, M., Yu, E. Y., Mostaghel, E. A., Cheng, H. H., Mulcahy, H., True, L. D., Plymate, S. R., Dvinge, H., Ferraldeschi, R., Flohr, P., Miranda, S., Zafeiriou, Z., Tunariu, N., Mateo, J., Perez-Lopez, R., Demichelis, F., Robinson, B. D., Schiffman, M., Nanus, D. M., Tagawa, S. T., Sigaras, A., Eng, K. W., Elemento, O., Sboner, A., Heath, E. I., Scher, H. I., Pienta, K. J., Kantoff, P., de Bono, J. S., Rubin, M. A., Nelson, P. S., Garraway, L. A., Sawyers, C. L. and Chinnaiyan, A. M. (2015) 'Integrative clinical genomics of advanced prostate cancer', *Cell,* 161(5), pp. 1215-1228.

Roehl, K. A., Han, M., Ramos, C. G., Antenor, J. A. and Catalona, W. J. (2004) 'Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: long-term results', *J Urol,* 172(3), pp. 910-4.

Roux, P. P., Ballif, B. A., Anjum, R., Gygi, S. P. and Blenis, J. (2004) 'Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase', *Proceedings of the National Academy of Sciences of the United States of America,* 101(37), pp. 13489-13494.

Russell, M. R., Nickerson, D. P. and Odorizzi, G. (2006) 'Molecular mechanisms of late endosome morphology, identity and sorting', *Curr Opin Cell Biol,* 18(4), pp. 422-8.

Saari, H., Lázaro-Ibáñez, E., Viitala, T., Vuorimaa-Laukkanen, E., Siljander, P. and Yliperttula, M. (2015) 'Microvesicle- and exosome-mediated drug delivery enhances the cytotoxicity of Paclitaxel in autologous prostate cancer cells', *Journal of Controlled Release,* 220, pp. 727-737.

Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P. and Snyder, S. H. (1994) 'RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs', *Cell,* 78(1), pp. 35-43.

Sahu, B., Laakso, M., Pihlajamaa, P., Ovaska, K., Sinielnikov, I., Hautaniemi, S. and Janne, O. A. (2013) 'FoxA1 specifies unique androgen and glucocorticoid receptor binding events in prostate cancer cells', *Cancer Res,* 73(5), pp. 1570-80.

Salmasi, A. H., Patel, N. and Kim, I. Y. (2016) 'Chapter 52 - Androgen Deprivation Therapy: Appropriate Patients, Timing to Initiate ADT, and Complications', in Mydlo, J.H. & Godec, C.J. (eds.) *Prostate Cancer (Second Edition)*. San Diego: Academic Press, pp. 481-489.

Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C., Bar-Peled, L. and Sabatini, D. M. (2008) 'The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1', *Science,* 320(5882), pp. 1496-501.

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2004) 'Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton', *Curr Biol,* 14(14), pp. 1296-302.

Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L. and Sabatini, D. M. (2006) 'Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB', *Mol Cell,* 22(2), pp. 159-68.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005) 'Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex', *Science,* 307(5712), pp. 1098-101.

Saxton, R. A., Knockenhauer, K. E., Schwartz, T. U. and Sabatini, D. M. (2016) 'The apo-structure of the leucine sensor Sestrin2 is still elusive', *Science Signaling,* 9(446), pp. ra92-ra92.

Schayowitz, A., Sabnis, G., Njar, V. C. and Brodie, A. M. (2008) 'Synergistic effect of a novel antiandrogen, VN/124-1, and signal transduction inhibitors in prostate cancer progression to hormone independence in vitro', *Mol Cancer Ther,* 7(1), pp. 121-32.

Schellhammer, P. F., Sharifi, R., Block, N. L., Soloway, M. S., Venner, P. M., Patterson, A. L., Sarosdy, M. F., Vogelzang, N. J., Chen, Y. and Kolvenbag, G. J. (1996) 'A controlled trial of bicalutamide versus flutamide, each in combination with luteinizing hormone-releasing hormone analogue therapy, in patients with advanced prostate carcinoma. Analysis of time to progression. CASODEX Combination Study Group', *Cancer,* 78(10), pp. 2164-9.

Secondini, C., Wetterwald, A., Schwaninger, R., Thalmann, G. N. and Cecchini, M. G. (2011) 'The role of the BMP signaling antagonist noggin in the development of prostate cancer osteolytic bone metastasis', *PLoS One,* 6(1), pp. e16078.

Settle, S., Marker, P., Gurley, K., Sinha, A., Thacker, A., Wang, Y., Higgins, K., Cunha, G. and Kingsley, D. M. (2001) 'The BMP Family Member Gdf7 Is Required for Seminal Vesicle Growth, Branching Morphogenesis, and Cytodifferentiation', *Developmental Biology,* 234(1), pp. 138-150.

Sharifi, N. (2013) 'Minireview: Androgen Metabolism in Castration-Resistant Prostate Cancer', *Molecular Endocrinology,* 27(5), pp. 708-714.

Sharma, A., Comstock, C. E. S., Knudsen, E. S., Cao, K. H., Hess-Wilson, J. K., Morey, L. M., Barrera, J. and Knudsen, K. E. (2007) 'Retinoblastoma Tumor Suppressor Status Is a Critical Determinant of Therapeutic Response in Prostate Cancer Cells', *Cancer research,* 67(13), pp. 6192-6203.

Sharma, A., Yeow, W. S., Ertel, A., Coleman, I., Clegg, N., Thangavel, C., Morrissey, C., Zhang, X., Comstock, C. E., Witkiewicz, A. K., Gomella, L., Knudsen, E. S., Nelson, P. S. and Knudsen, K. E. (2010) 'The retinoblastoma tumor suppressor controls androgen signaling and human prostate cancer progression', *J Clin Invest,* 120(12), pp. 4478-92.

Shi, Y., Yan, H., Frost, P., Gera, J. and Lichtenstein, A. (2005) 'Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the

insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade', *Molecular Cancer Therapeutics,* 4(10), pp. 1533-1540.

Shiozawa, Y., Pedersen, E. A., Havens, A. M., Jung, Y., Mishra, A., Joseph, J., Kim, J. K., Patel, L. R., Ying, C., Ziegler, A. M., Pienta, M. J., Song, J., Wang, J., Loberg, R. D., Krebsbach, P. H., Pienta, K. J. and Taichman, R. S. (2011) 'Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow', *The Journal of Clinical Investigation,* 121(4), pp. 1298-1312.

Simanainen, U., Allan, C. M., Lim, P., McPherson, S., Jimenez, M., Zajac, J. D., Davey, R. A. and Handelsman, D. J. (2007) 'Disruption of prostate epithelial androgen receptor impedes prostate lobe-specific growth and function', *Endocrinology,* 148(5), pp. 2264-72.

Soekmadji, C., Riches, J. D., Russell, P. J., Ruelcke, J. E., McPherson, S., Wang, C., Hovens, C. M., Corcoran, N. M., The Australian Prostate Cancer Collaboration, B., Hill, M. M. and Nelson, C. C. (2017) 'Modulation of paracrine signaling by CD9 positive small extracellular vesicles mediates cellular growth of androgen deprived prostate cancer', *Oncotarget,* 8(32), pp. 52237-52255.

Somdyala, N. I. M., Parkin, D. M., Sithole, N. and Bradshaw, D. (2014) 'Trends in cancer incidence in rural Eastern Cape Province; South Africa, 1998–2012', *International Journal of Cancer,* 136(5), pp. E470-E474.

Sosa, M. S., Bragado, P. and Aguirre-Ghiso, J. A. (2014) 'Mechanisms of disseminated cancer cell dormancy: an awakening field', *Nature reviews. Cancer,* 14(9), pp. 611-622.

Spanjol, J., Djordjevic, G., Markic, D., Klaric, M., Fuckar, D. and Bobinac, D. (2010) 'Role of bone morphogenetic proteins in human prostate cancer pathogenesis and development of bone metastases: immunohistochemical study', *Coll Antropol,* 34 Suppl 2, pp. 119-25.

Stanbrough, M., Bubley, G. J., Ross, K., Golub, T. R., Rubin, M. A., Penning, T. M., Febbo, P. G. and Balk, S. P. (2006) 'Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer', *Cancer Res,* 66(5), pp. 2815-25.

Staneva, G., Momchilova, A., Wolf, C., Quinn, P. J. and Koumanov, K. (2009) 'Membrane microdomains: Role of ceramides in the maintenance of their structure and functions', *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1788(3), pp. 666-675.

Statz, C. M., Patterson, S. E. and Mockus, S. M. (2017) 'mTOR Inhibitors in Castration-Resistant Prostate Cancer: A Systematic Review', *Target Oncol,* 12(1), pp. 47-59.

Steinsvik, E. A., Fosså, S. D., Lilleby, W. and Eilertsen, K. (2008) 'Fertility issues in patients with prostate cancer', *BJU International,* 102(7), pp. 793-795.

Steketee, K., Timmerman, L., Ziel-van der Made, A. C., Doesburg, P., Brinkmann, A. O. and Trapman, J. (2002) 'Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer', *Int J Cancer,* 100(3), pp. 309-17.

Stuffers, S., Sem Wegner, C., Stenmark, H. and Brech, A. (2009) 'Multivesicular Endosome Biogenesis in the Absence of ESCRTs', *Traffic,* 10(7), pp. 925-937.

Szmulewitz, R. Z., Chung, E., Al-Ahmadie, H., Daniel, S., Kocherginsky, M., Razmaria, A., Zagaja, G. P., Brendler, C. B., Stadler, W. M. and Conzen, S. D. (2011) 'Serum/glucocorticoid-regulated kinase 1 expression in primary human prostate cancers', *The Prostate,* 72(2), pp. 157-164.

Tamai, K., Tanaka, N., Nakano, T., Kakazu, E., Kondo, Y., Inoue, J., Shiina, M., Fukushima, K., Hoshino, T., Sano, K., Ueno, Y., Shimosegawa, T. and Sugamura, K. (2010) 'Exosome

secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein', *Biochemical and Biophysical Research Communications,* 399(3), pp. 384-390.

Tannock, I. F., de Wit, R., Berry, W. R., Horti, J., Pluzanska, A., Chi, K. N., Oudard, S., Theodore, C., James, N. D., Turesson, I., Rosenthal, M. A. and Eisenberger, M. A. (2004) 'Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer', *N Engl J Med,* 351(15), pp. 1502-12.

Taplin, M. E., Bubley, G. J., Shuster, T. D., Frantz, M. E., Spooner, A. E., Ogata, G. K., Keer, H. N. and Balk, S. P. (1995) 'Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer', *N Engl J Med,* 332(21), pp. 1393-8.

Tauro, B. J., Greening, D. W., Mathias, R. A., Ji, H., Mathivanan, S., Scott, A. M. and Simpson, R. J. (2012) 'Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes', *Methods,* 56(2), pp. 293-304.

Taylor, B. S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B. S., Arora, V. K., Kaushik, P., Cerami, E., Reva, B., Antipin, Y., Mitsiades, N., Landers, T., Dolgalev, I., Major, J. E., Wilson, M., Socci, N. D., Lash, A. E., Heguy, A., Eastham, J. A., Scher, H. I., Reuter, V. E., Scardino, P. T., Sander, C., Sawyers, C. L., Gerald, W. L. and for the, M. P. C. O. G. (2010) 'Integrative genomic profiling of human prostate cancer', *Cancer cell,* 18(1), pp. 11-22.

Templeton, A. J., Dutoit, V., Cathomas, R., Rothermundt, C., Bartschi, D., Droge, C., Gautschi, O., Borner, M., Fechter, E., Stenner, F., Winterhalder, R., Muller, B., Schiess, R., Wild, P. J., Ruschoff, J. H., Thalmann, G., Dietrich, P. Y., Aebersold, R., Klingbiel, D. and Gillessen, S. (2013) 'Phase 2 trial of single-agent everolimus in chemotherapy-naive patients with castration-resistant prostate cancer (SAKK 08/08)', *Eur Urol,* 64(1), pp. 150-8.

Tenniswood, M. P., Abrahams, P. P., Bird, C. E. and Clark, A. F. (1982) 'Age-Associated Changes in Acid Phosphatase Characteristics in the Rat Ventral Prostate and Other Organs', *Archives of Andrology,* 9(4), pp. 283-291.

Thangavel, C., Boopathi, E., Liu, Y., Haber, A., Ertel, A., Bhardwaj, A., Addya, S., Williams, N., Ciment, S. J., Cotzia, P., Dean, J. L., Snook, A., McNair, C., Price, M., Hernandez, J. R., Zhao, S. G., Birbe, R., McCarthy, J. B., Turley, E. A., Pienta, K. J., Feng, F. Y., Dicker, A. P., Knudsen, K. E. and Den, R. B. (2017) 'RB Loss Promotes Prostate Cancer Metastasis', *Cancer research,* 77(4), pp. 982-995.

Thery, C., Amigorena, S., Raposo, G. and Clayton, A. (2006) 'Isolation and characterization of exosomes from cell culture supernatants and biological fluids', *Curr Protoc Cell Biol,* Chapter 3, pp. Unit 3.22.

Théry, C., Boussac, M., Véron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J. and Amigorena, S. (2001) 'Proteomic Analysis of Dendritic Cell-Derived Exosomes: A Secreted Subcellular Compartment Distinct from Apoptotic Vesicles', *The Journal of Immunology,* 166(12), pp. 7309-7318.

Thomas, C., Lamoureux, F., Crafter, C., Davies, B. R., Beraldi, E., Fazli, L., Kim, S., Thaper, D., Gleave, M. E. and Zoubeidi, A. (2013) 'Synergistic targeting of PI3K/AKT pathway and androgen receptor axis significantly delays castration-resistant prostate cancer progression in vivo', *Mol Cancer Ther,* 12(11), pp. 2342-55.

Thomas, J. D., Zhang, Y. J., Wei, Y. H., Cho, J. H., Morris, L. E., Wang, H. Y. and Zheng, X. F. (2014) 'Rab1A is an mTORC1 activator and a colorectal oncogene', *Cancer Cell,* 26(5), pp. 754-69.

Thomas, R., True, L. D., Lange, P. H. and Vessella, R. L. (2001) 'Placental bone morphogenetic protein (PLAB) gene expression in normal, pre-malignant and malignant human prostate: Relation to tumor development and progression', *International Journal of Cancer,* 93(1), pp. 47-52.

Thoreen, C. C., Kang, S. A., Chang, J. W., Liu, Q., Zhang, J., Gao, Y., Reichling, L. J., Sim, T., Sabatini, D. M. and Gray, N. S. (2009) 'An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1', *J Biol Chem,* 284(12), pp. 8023-32.

Thorpe, L. M., Yuzugullu, H. and Zhao, J. J. (2015) 'PI3K in cancer: divergent roles of isoforms, modes of activation, and therapeutic targeting', *Nature reviews. Cancer,* 15(1), pp. 7-24.

Titus, M. A., Schell, M. J., Lih, F. B., Tomer, K. B. and Mohler, J. L. (2005) 'Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer', *Clin Cancer Res,* 11(13), pp. 4653-7.

Todorova, V. K., Kaufmann, Y., Luo, S. and Suzanne Klimberg, V. (2011) 'Tamoxifen and raloxifene suppress the proliferation of estrogen receptor-negative cells through inhibition of glutamine uptake', *Cancer Chemotherapy and Pharmacology,* 67(2), pp. 285-291.

Traish, A. M. and Morgentaler, A. (2009) 'Epidermal growth factor receptor expression escapes androgen regulation in prostate cancer: a potential molecular switch for tumour growth', *British Journal of Cancer,* 101(12), pp. 1949-1956.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B. and Simons, M. (2008) 'Ceramide triggers budding of exosome vesicles into multivesicular endosomes', *Science,* 319(5867), pp. 1244-7.

Trams, E. G., Lauter, C. J., Norman Salem, Jr. and Heine, U. (1981) 'Exfoliation of membrane ecto-enzymes in the form of micro-vesicles', *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 645(1), pp. 63-70.

Trotman, L. C., Niki, M., Dotan, Z. A., Koutcher, J. A., Di Cristofano, A., Xiao, A., Khoo, A. S., Roy-Burman, P., Greenberg, N. M., Dyke, T. V., Cordon-Cardo, C. and Pandolfi, P. P. (2003) 'Pten Dose Dictates Cancer Progression in the Prostate', *PLOS Biology,* 1(3), pp. e59.

Tsouko, E., Khan, A. S., White, M. A., Han, J. J., Shi, Y., Merchant, F. A., Sharpe, M. A., Xin, L. and Frigo, D. E. (2014) 'Regulation of the pentose phosphate pathway by an androgen receptor–mTOR-mediated mechanism and its role in prostate cancer cell growth', *Oncogenesis,* 3(5), pp. e103.

Tsun, Z. Y., Bar-Peled, L., Chantranupong, L., Zoncu, R., Wang, T., Kim, C., Spooner, E. and Sabatini, D. M. (2013) 'The folliculin tumor suppressor is a GAP for the RagC/D GTPases that signal amino acid levels to mTORC1', *Mol Cell,* 52(4), pp. 495-505.

Tuxhorn, J. A., Ayala, G. E. and Rowley, D. R. (2001) 'Reactive stroma in prostate cancer progression', *J Urol,* 166(6), pp. 2472-83.

Ueda, T., Mawji, N. R., Bruchovsky, N. and Sadar, M. D. (2002) 'Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells', *J Biol Chem,* 277(41), pp. 38087-94.

UK, C. R. (2016): Cancer Research. Available at: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer/incidence.

van Niel, G., Charrin, S., Simoes, S., Romao, M., Rochin, L., Saftig, P., Marks, M. S., Rubinstein, E. and Raposo, G. (2011) 'The tetraspanin CD63 regulates ESCRT-independent and dependent endosomal sorting during melanogenesis', *Developmental cell,* 21(4), pp. 708-721.

Velasco, A. M., Gillis, K. A., Li, Y., Brown, E. L., Sadler, T. M., Achilleos, M., Greenberger, L. M., Frost, P., Bai, W. and Zhang, Y. (2004) 'Identification and validation of novel androgen-regulated genes in prostate cancer', *Endocrinology,* 145(8), pp. 3913-24.

Vidal, M. J. and Stahl, P. D. (1993) 'The small GTP-binding proteins Rab4 and ARF are associated with released exosomes during reticulocyte maturation', *Eur J Cell Biol,* 60(2), pp. 261-7.

Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinanen, R., Palmberg, C., Palotie, A., Tammela, T., Isola, J. and Kallioniemi, O. P. (1995) 'In vivo amplification of the androgen receptor gene and progression of human prostate cancer', *Nat Genet,* 9(4), pp. 401-6.

Vlietstra, R. J., van Alewijk, D. C., Hermans, K. G., van Steenbrugge, G. J. and Trapman, J. (1998) 'Frequent inactivation of PTEN in prostate cancer cell lines and xenografts', *Cancer Res,* 58(13), pp. 2720-3.

Waghray, A., Feroze, F., Schober, M. S., Yao, F., Wood, C., Puravs, E., Krause, M., Hanash, S. and Chen, Y. Q. (2001) 'Identification of androgen-regulated genes in the prostate cancer cell line LNCaP by serial analysis of gene expression and proteomic analysis', *Proteomics,* 1(10), pp. 1327-38.

Wahdan-Alaswad, R. S., Bane, K. L., Song, K., Shola, D. T., Garcia, J. A. and Danielpour, D. (2012) 'Inhibition of mTORC1 kinase activates Smads 1 and 5 but not Smad8 in human prostate cancer cells, mediating cytostatic response to rapamycin', *Mol Cancer Res,* 10(6), pp. 821-33.

Wallen, M. J., Linja, M., Kaartinen, K., Schleutker, J. and Visakorpi, T. (1999) 'Androgen receptor gene mutations in hormone-refractory prostate cancer', *J Pathol,* 189(4), pp. 559-63.

Waltering, K. K., Helenius, M. A., Sahu, B., Manni, V., Linja, M. J., Janne, O. A. and Visakorpi, T. (2009) 'Increased expression of androgen receptor sensitizes prostate cancer cells to low levels of androgens', *Cancer Res,* 69(20), pp. 8141-9.

Wang, Q., Bailey, C. G., Ng, C., Tiffen, J., Thoeng, A., Minhas, V., Lehman, M. L., Hendy, S. C., Buchanan, G., Nelson, C. C., Rasko, J. E. and Holst, J. (2011) 'Androgen receptor and nutrient signaling pathways coordinate the demand for increased amino acid transport during prostate cancer progression', *Cancer Res,* 71(24), pp. 7525-36.

Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G. V., Li, G., Roy-Burman, P., Nelson, P. S., Liu, X. and Wu, H. (2003) 'Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer', *Cancer Cell,* 4(3), pp. 209-221.

Wang, S., Tsun, Z. Y., Wolfson, R. L., Shen, K., Wyant, G. A., Plovanich, M. E., Yuan, E. D., Jones, T. D., Chantranupong, L., Comb, W., Wang, T., Bar-Peled, L., Zoncu, R., Straub, C., Kim, C., Park, J., Sabatini, B. L. and Sabatini, D. M. (2015) 'Metabolism. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1', *Science,* 347(6218), pp. 188-94.

Wang, Y., Mikhailova, M., Bose, S., Pan, C. X., deVere White, R. W. and Ghosh, P. M. (2008) 'Regulation of androgen receptor transcriptional activity by rapamycin in prostate cancer cell proliferation and survival', *Oncogene,* 27(56), pp. 7106-17.

Wen, Y., Hu, M. C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D. H. and Hung, M. C. (2000) 'HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway', *Cancer Res,* 60(24), pp. 6841-5.

White, I. J., Bailey, L. M., Aghakhani, M. R., Moss, S. E. and Futter, C. E. (2006) 'EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation', *Embo j,* 25(1), pp. 1-12.

White, M. A., Lin, C., Rajapakshe, K., Dong, J., Shi, Y., Tsouko, E., Mukhopadhyay, R., Jasso, D., Dawood, W., Coarfa, C. and Frigo, D. E. (2017) 'Glutamine Transporters Are Targets of Multiple Oncogenic Signaling Pathways in Prostate Cancer', *Mol Cancer Res,* 15(8), pp. 1017-1028.

Williams, N., Hughes, L. J., Turner, E. L., Donovan, J. L., Hamdy, F. C., Neal, D. E., Martin, R. M. and Metcalfe, C. (2011) 'Prostate-specific antigen testing rates remain low in UK general practice: a cross-sectional study in six English cities', *BJU Int,* 108(9), pp. 1402-8.

Willis, G. R., Kourembanas, S. and Mitsialis, S. A. (2017) 'Toward Exosome-Based Therapeutics: Isolation, Heterogeneity, and Fit-for-Purpose Potency', *Frontiers in Cardiovascular Medicine,* 4, pp. 63.

Willms, E., Cabañas, C., Mäger, I., Wood, M. J. A. and Vader, P. (2018) 'Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression', *Frontiers in Immunology,* 9, pp. 738.

Willms, E., Johansson, H. J., Mäger, I., Lee, Y., Blomberg, K. E. M., Sadik, M., Alaarg, A., Smith, C. I. E., Lehtiö, J., El Andaloussi, S., Wood, M. J. A. and Vader, P. (2016) 'Cells release subpopulations of exosomes with distinct molecular and biological properties', *Scientific Reports,* 6, pp. 22519.

Wilson, C., Leiblich, A., Goberdhan, D. C. I. and Hamdy, F. (2017) 'Chapter Eleven: The Drosophila Accessory Gland as a Model for Prostate Cancer and Other Pathologies', *Current topics in developmental biology,* 121, pp. 339-375.

Witkiewicz, A. K., and Knudsen, E. S. (2014). 'Retinoblastoma tumor suppressor pathway in breast cancer: prognosis, precision medicine, and therapeutic interventions'. *Breast Cancer Research : BCR*, *16*(3), pp. 207.

Wolf, I. M., Heitzer, M. D., Grubisha, M. and DeFranco, D. B. (2008) 'Coactivators and nuclear receptor transactivation', *Journal of Cellular Biochemistry,* 104(5), pp. 1580-1586.

Wolf, P. (1967) 'The Nature and Significance of Platelet Products in Human Plasma', *British Journal of Haematology,* 13(3), pp. 269-288.

Wolfson, R. L., Chantranupong, L., Saxton, R. A., Shen, K., Scaria, S. M., Cantor, J. R. and Sabatini, D. M. (2016) 'Sestrin2 is a leucine sensor for the mTORC1 pathway', *Science (New York, N.Y.),* 351(6268), pp. 43-48.

Wollert, T., Wunder, C., Lippincott-Schwartz, J. and Hurley, J. H. (2009) 'Membrane scission by the ESCRT-III complex', *Nature,* 458(7235), pp. 172-7.

Wong, M. C., Goggins, W. B., Wang, H. H., Fung, F. D., Leung, C., Wong, S. Y., Ng, C. F. and Sung, J. J. (2016) 'Global Incidence and Mortality for Prostate Cancer: Analysis of Temporal Patterns and Trends in 36 Countries', *Eur Urol,* 70(5), pp. 862-874.

World Helath Organisation, National Institute on Ageing, National Institutes of Health, & U.S. Department of Health and Human Services. (2011). Global Health and Ageing (NIH Publication no. 11-7737). Bethesda, MD: National Institute of Health.

Wozney, J. M. (2002) 'Overview of bone morphogenetic proteins', *Spine (Phila Pa 1976),* 27(16 Suppl 1), pp. S2-8.

Wu, Y., Zhao, W., Zhao, J., Pan, J., Wu, Q., Zhang, Y., Bauman, W. A. and Cardozo, C. P. (2007) 'Identification of androgen response elements in the insulin-like growth factor I upstream promoter', *Endocrinology,* 148(6), pp. 2984-93.

Wu, Y. U. E., Chhipa, R. R., Cheng, J., Zhang, H., Mohler, J. L. and Clement, I. P. (2010) 'Androgen Receptor-mTOR Crosstalk is Regulated by Testosterone Availability: Implication for Prostate Cancer Cell Survival', *Anticancer research,* 30(10), pp. 3895-3901.

Xin, L., Teitell, M. A., Lawson, D. A., Kwon, A., Mellinghoff, I. K. and Witte, O. N. (2006) 'Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor', *Proceedings of the National Academy of Sciences of the United States of America,* 103(20), pp. 7789-7794.

Xu, K., Shimelis, H., Linn, D. E., Jiang, R., Yang, X., Sun, F., Guo, Z., Chen, H., Li, W., Chen, H., Kong, X., Melamed, J., Fang, S., Xiao, Z., Veenstra, T. D. and Qiu, Y. (2009) 'Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination', *Cancer Cell,* 15(4), pp. 270-82.

Xu, R., Greening, D. W., Rai, A., Ji, H. and Simpson, R. J. (2015) 'Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct', *Methods,* 87, pp. 11-25.

Xu, Y., Chen, S. Y., Ross, K. N. and Balk, S. P. (2006) 'Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins', *Cancer Res,* 66(15), pp. 7783-92.

Xue, Y., Sonke, G., Schoots, C., Schalken, J., Verhofstad, A., de la Rosette, J. and Smedts, F. (2001) 'Proliferative activity and branching morphogenesis in the human prostate: a closer look at pre- and postnatal prostate growth', *Prostate,* 49(2), pp. 132-9.

Yang, F., Chen, Y., Shen, T., Guo, D., Dakhova, O., Ittmann, M. M., Creighton, C. J., Zhang, Y., Dang, T. D. and Rowley, D. R. (2014) 'Stromal TGF-β signaling induces AR activation in prostate cancer', *Oncotarget,* 5(21), pp. 10854-10869.

Yang, S., Lim, M., Pham, L. K., Kendall, S. E., Reddi, A. H., Altieri, D. C. and Roy-Burman, P. (2006) 'Bone morphogenetic protein 7 protects prostate cancer cells from stress-induced apoptosis via both Smad and c-Jun NH2-terminal kinase pathways', *Cancer Res,* 66(8), pp. 4285-90.

Yang, S., Zhong, C., Frenkel, B., Reddi, A. H. and Roy-Burman, P. (2005) 'Diverse biological effect and Smad signaling of bone morphogenetic protein 7 in prostate tumor cells', *Cancer Res,* 65(13), pp. 5769-77.

Yepuru, M., Wu, Z., Kulkarni, A., Yin, F., Barrett, C. M., Kim, J., Steiner, M. S., Miller, D. D., Dalton, J. T. and Narayanan, R. (2013) 'Steroidogenic Enzyme AKR1C3 Is a Novel Androgen Receptor-Selective Coactivator that Promotes Prostate Cancer Growth', *Clinical Cancer Research,* 19(20), pp. 5613-5625.

Zabkiewicz, C., Resaul, J., Hargest, R., Jiang, W. G. and Ye, L. (2017) 'Bone morphogenetic proteins, breast cancer, and bone metastases: striking the right balance', *Endocrine-Related Cancer,* 24(10), pp. R349-R366.

Zhang, C., Soori, M., Miles, F., Sikes, R. A., Carson, D. D., Chung, L. L. W. and Farach-Carson, M. C. (2011) 'Paracrine Factors Produced by Bone Marrow Stromal Cells Induce

Apoptosis and Neuroendocrine Differentiation in Prostate Cancer Cells', *The Prostate,* 71(2), pp. 157-167.

Zhang, H., Freitas, D., Kim, H. S., Fabijanic, K., Li, Z., Chen, H., Mark, M. T., Molina, H., Martin, A. B., Bojmar, L., Fang, J., Rampersaud, S., Hoshino, A., Matei, I., Kenific, C. M., Nakajima, M., Mutvei, A. P., Sansone, P., Buehring, W., Wang, H., Jimenez, J. P., Cohen-Gould, L., Paknejad, N., Brendel, M., Manova-Todorova, K., Magalhaes, A., Ferreira, J. A., Osorio, H., Silva, A. M., Massey, A., Cubillos-Ruiz, J. R., Galletti, G., Giannakakou, P., Cuervo, A. M., Blenis, J., Schwartz, R., Brady, M. S., Peinado, H., Bromberg, J., Matsui, H., Reis, C. A. and Lyden, D. (2018) 'Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation', *Nat Cell Biol,* 20(3), pp. 332-343.

Zhao, H., Yang, L., Baddour, J., Achreja, A., Bernard, V., Moss, T., Marini, J. C., Tudawe, T., Seviour, E. G., San Lucas, F. A., Alvarez, H., Gupta, S., Maiti, S. N., Cooper, L., Peehl, D., Ram, P. T., Maitra, A. and Nagrath, D. (2016) 'Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism', *Elife,* 5, pp. e10250.

Zhu, M. L. and Kyprianou, N. (2008) 'Androgen receptor and growth factor signaling cross-talk in prostate cancer cells', *Endocr Relat Cancer,* 15(4), pp. 841-9.

Zoncu, R., Efeyan, A. and Sabatini, D. M. (2011) 'mTOR: from growth signal integration to cancer, diabetes and ageing', *Nat Rev Mol Cell Biol,* 12(1), pp. 21-35.