

**Novel signalling pathways regulating
epithelial-mesenchymal transition in bone metastatic
prostate cancer**

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*A thesis submitted for the degree of
Doctor of Philosophy*

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Abstract

Prostate cancer (PCa) cells predominantly metastasize to bone and the complex crosstalk between PCa cells and osteoblasts (bone-forming cells) and osteoclasts (bone-destroying cells) leads to increased tumour growth and worsening of bone disease. Understanding the mechanisms of PCa bone metastasis can identify the aggressive fraction of PCa resulting in earlier intervention. The ability of PCa cells to express bone cell-specific features, termed osteomimicry, could potentially explain the osteotropic nature of PCa cells. The aim of this study was to determine the role of osteomimicry in the regulation of epithelial-mesenchymal transition (EMT) in bone metastatic prostate cancer cells.

It was demonstrated that the osteoblast-specific marker alkaline phosphatase (bone/liver/kidney) (ALPL) was overexpressed in bone metastatic (ARCaPM), compared to non-metastatic (ARCaPE), human PCa cells. Knockdown of ALPL resulted in decreased cell viability, increased cell death and a change from mesenchymal to epithelial morphology in ARCaPM and PC3 cells, and increased CDH1 expression along with decreased migration in ARCaPM cells. Treatment with extracellular ATP also resulted in decreased viability, increased expression of epithelial markers (CDH1, KRT14) and decreased expression of mesenchymal markers (VIM, ZEB1), and reduced expression of ALPL in ARCaPM cells. Small RNA-sequencing identified microRNAs differentially expressed between ARCaPE and ARCaPM PCa cell lines: miR-373 expression was lower in ARCaPM compared to ARCaPE cells and its overexpression in ARCaPM cells resulted in a change to epithelial morphology, increased expression of the epithelial marker CDH1 and decreased expression of the mesenchymal markers VIM and ZEB1. Finally, the development of a high-throughput screening method to identify novel microRNA regulators of osteomimicry was described, which identified two microRNAs miR-199a-5p and miR-212 as positive regulators of ALP activity. Taken together, this thesis describes the identification of ALPL and ATP as novel regulators of epithelial-mesenchymal transition in PCa cells and high-throughput ALP-activity screening as a powerful tool to identify novel microRNA regulators of ALP expression.

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*Answers are like opium: the more you imbibe, the more
you need. Which is why the sober man finds solace in
mystery.*

—R. Scott Bakker
The Darkness That Comes Before

Acknowledgements

Oh I get by with a little help from my friends...

—John Lennon and Paul McCartney
Sgt. Pepper's Lonely Hearts Club Band

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Statement of Originality

I affirm that all the data presented in this thesis are from experiments performed and analysed by me, unless specifically attributed to someone else. All figures adapted or reproduced herein were used with express permission or under freely distributable copyright (Creative Commons copyright code is mentioned in figure legends where necessary) or are in the public domain.

Abstract

Prostate cancer (PCa) cells predominantly metastasize to bone and the complex crosstalk between PCa cells and osteoblasts (bone-forming cells) and osteoclasts (bone-destroying cells) leads to increased tumour growth and worsening of bone disease. Understanding the mechanisms of PCa bone metastasis can identify the aggressive fraction of PCa resulting in earlier intervention. The ability of PCa cells to express bone cell-specific features, termed osteomimicry, could potentially explain the osteotropic nature of PCa cells. The aim of this study was to determine the role of osteomimicry in the regulation of epithelial-mesenchymal transition (EMT) in bone metastatic prostate cancer cells.

It was demonstrated that the osteoblast-specific marker alkaline phosphatase (bone/liver/kidney) (ALPL) was overexpressed in bone metastatic (ARCaPM), compared to non-metastatic (ARCaPE), human PCa cells. Knockdown of ALPL resulted in decreased cell viability, increased cell death and a change from mesenchymal to epithelial morphology in ARCaPM and PC3 cells, and increased CDH1 expression along with decreased migration in ARCaPM cells. Treatment with extracellular ATP also resulted in decreased viability, increased expression of epithelial markers (CDH1, KRT14) and decreased expression of mesenchymal markers (VIM, ZEB1), and reduced expression of ALPL in ARCaPM cells. Small RNA-sequencing identified microRNAs differentially expressed between ARCaPE and ARCaPM PCa cell lines: miR-373 expression was lower in ARCaPM compared to ARCaPE cells and its overexpression in ARCaPM cells resulted in a change to epithelial morphology, increased expression of the epithelial marker CDH1 and decreased expression of the mesenchymal markers VIM and ZEB1. Finally, the development of a high-throughput screening method to identify novel microRNA regulators of osteomimicry was described, which identified two microRNAs miR-199a-5p and miR-212 as positive regulators of ALP activity. Taken together, this thesis describes the identification of ALPL and ATP as novel regulators of epithelial-mesenchymal transition in PCa cells and high-throughput ALP-activity screening as a powerful tool to identify novel microRNA regulators of ALP expression.

Contents

List of Figures	viii
List of Tables	xii
Abbreviations	xiv
1 Introduction	1
1.1 Introduction	1
1.1.1 History and epidemiology	2
1.1.2 Anatomy and physiology of the prostate gland	6
1.1.3 Prostate tumourigenesis	7
1.1.4 Clinical features	8
1.1.5 Treatment and remission	12
1.1.6 Metastasis in cancer	13
1.1.7 Bone metastasis in prostate cancer	14
1.2 The bone microenvironment	14
1.2.1 Osteoblasts	15
1.2.2 Osteoclasts	18
1.2.3 Interaction of prostate cancer cells with the bone micro- environment	18
1.3 Factors with a role in prostate cancer progression and bone metastasis	19

1.3.1	Androgen receptor	19
1.3.2	PTEN	21
1.3.3	TNFSF11/RANKL	21
1.3.4	Endothelin-1	21
1.3.5	Runx2	22
1.3.6	Parathyroid Hormone-related Peptide (PTHrP)	22
1.3.7	Dickkopf-1 (DKK1)	23
1.3.8	Matrix metalloproteinases	23
1.3.9	uPA and uPAR	23
1.3.10	Growth factors	24
1.4	Epithelial-Mesenchymal Transition	27
1.4.1	Key proteins in EMT	29
1.4.2	Signalling pathways regulating EMT	31
1.4.3	Role of EMT in bone metastasis	32
1.4.4	ARCaP cell line model of EMT	33
1.5	MicroRNAs	34
1.5.1	Biogenesis	34
1.5.2	Function	35
1.5.3	MicroRNAs in prostate cancer	36
1.5.4	MicroRNAs regulating EMT in prostate cancer	36
1.6	Prostate cancer animal models	37
1.6.1	Tumourigenesis models	37
1.6.2	Xenograft models	37
1.7	Osteomimicry	40
1.8	Hypothesis and aims	40

2	Materials and Methods	42
2.1	Cell lines and reagents	42
2.2	Transfection	43
2.2.1	Plasmid DNA transfection	43
2.2.2	siRNA and microRNA mimic transfection	43
2.3	Lentiviral packaging and transduction	44
2.4	RNA isolation	45
2.5	Real-time quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)	46
2.6	Protein isolation and western blotting	48
2.7	Cell viability	49
2.8	YO-PRO-1 staining	49
2.9	Alkaline Phosphatase (ALP) activity assays	50
2.9.1	Staining for alkaline phosphatase activity	50
2.9.2	Quantitative measurement of alkaline phosphatase activity	50
2.10	Mineralization assay	51
2.11	Small RNA-sequencing	51
2.12	Statistics	51
3	ALPL regulates EMT and cell survival in prostate cancer cells	53
3.1	Introduction	53
3.1.1	Osteomimicry	53
3.1.2	<i>In vitro</i> mineralization in prostate cancer cells	54
3.1.3	Alkaline phosphatase (bone/liver/kidney) (ALPL)	55
3.2	Aim	55
3.3	Methods	55
3.3.1	<i>In vitro</i> migration assay	55

3.3.2	Data mining	56
3.4	Results	56
3.4.1	ARCaPE/M cells are a model of epithelial-mesenchymal transition	56
3.4.2	ARCaPM cells express high levels of ALPL	58
3.4.3	Differential expression of ALPL in a panel of prostate cell lines	59
3.4.4	ARCaPM cells, but not ARCaPE, mineralize <i>in vitro</i>	62
3.4.5	Knockdown of ALPL induces change in morphology and induces cell death in ARCaPM cells	64
3.4.6	Knockdown of ALPL reduces migration in ARCaPM	67
3.4.7	ALPL knockdown results in increased expression of E-cadherin	68
3.4.8	Knockdown of ALPL induces change in morphology, reduction in viability and increased cell death in PC3 cells	68
3.4.9	Inhibition of ALP activity with levamisole decreases cell viability and induces cell death in ARCaPM cells	71
3.4.10	Snail knockdown in ARCaPM cells results in decreased ALP activity	73
3.4.11	Alkaline phosphatase expression correlates positively with Snail and negatively with PTEN expression in patients with prostate cancer	75
3.5	Summary	77
3.6	Conclusions	77
3.6.1	ARCaP model	78
3.6.2	<i>In vitro</i> mineralization and ALP activity	78
3.6.3	Role of ALPL in EMT	79
3.6.4	Limitations and further studies	81

4	ATP induces MET in prostate cancer cells	83
4.1	Introduction	83
4.1.1	ATP	83
4.1.2	Purinergic signalling in cancer	84
4.1.3	Expression and function of P2 receptors in prostate cancer cells	85
4.2	Hypothesis and aim	85
4.3	Methods	87
4.3.1	High-throughput, automated quantitation of changes in cell shape	87
4.3.2	ATP assay	87
4.4	Results	88
4.4.1	Exposure to ATP results in a change in morphology and reduced viability in ARCaPM cells	88
4.4.2	ATP increases epithelial marker and decreases mesenchymal marker gene expression in ARCaPM cells	90
4.4.3	The effects of ATP are greater than other purinergic agonists in ARCaPM cells	92
4.4.4	High-throughput (automated) quantitation of cell morphology	93
4.4.5	Non-specific blockade of purinergic receptors has no effect on ARCaPM cells' response to ATP	95
4.4.6	Expression of the P2X7 purinergic receptor in prostate cancer cell lines	96
4.4.7	Apyrase does not rescue ARCaPM cells from the effects of ALPL knockdown	97
4.4.8	ATP causes a reduction in alkaline phosphatase expression and activity in ARCaPM cells	98

4.5	Summary	99
4.6	Conclusions	99
4.6.1	Role in MET and involvement of specific purinergic receptors	100
4.6.2	ATP and ALP	101
4.6.3	Limitations and future studies	102
5	MicroRNAs regulate EMT and alkaline phosphatase expression	104
5.1	Introduction	104
5.1.1	MicroRNAs in prostate cancer bone metastasis	104
5.1.2	MicroRNAs regulating EMT	105
5.2	Hypothesis and aims	105
5.3	Methods	106
5.3.1	Establishment of miR-203 overexpressing stable cell lines . .	106
5.3.2	MicroRNA mimic library pilot screening	106
5.4	Results	107
5.4.1	miR-203 inhibits osteomimicry in ARCaPM cells	107
5.4.2	Small RNA-sequencing: differential expression of microR- NAs between ARCaPE and ARCaPM cells	110
5.4.3	Validation of RNA-seq: miR-373 is differentially expressed in ARCaPE vs ARCaPM and induces a mesenchymal-epithelial transition	115
5.4.4	Functional screening of a microRNA mimic library for ALP activity: pilot study identified microRNAs that regulate ALP activity	118
5.4.5	miR-199a-5p upregulates ALP activity in ARCaPM cells . .	121
5.5	Summary	123
5.6	Conclusions	124

5.6.1	Limitations and future studies	126
6	Conclusions and significance	128
A	Appendix	136
A.1	Levamisole treatment decreased 22Rv1 cell viability	136
A.2	ALPL expression is weakly negatively correlated with E-cadherin and ZEB1 expression in human prostate cancer samples	137
A.3	Apyrase degrades ATP <i>in vitro</i>	138
A.4	Apyrase does not rescue ARCaPM cells from levamisole-induced decrease in viability	139
A.5	Exogenously added porcine ALP has ecto-nucleotidase function <i>in</i> <i>vitro</i> , but has no effect on ARCaPE cell viability	140
A.6	Optimisation of mimic library screen	141
B	Appendix	142
B.1	R code for data mining	142
B.2	Pyridoxine concentration in growth media	143
C	Appendix	144
	Bibliography	146

List of Figures

1.1	Andreas Vesalius (1514-1564) first illustrated the prostate gland . . .	3
1.2	UK Statistics for 20 most common cancers	5
1.3	Clinical features of prostate cancer	7
1.4	Bone metastasis in prostate cancer	9
1.5	Steps involved in metastasis of cancer cells to distant sites	13
1.6	Factors expressed by an osteoblast	16
1.7	Prostate cancer cells cross-talk with bone cells	20
1.8	Epithelial-Mesenchymal Transition	29
1.9	Xenograft mouse models of prostate cancer	39
3.1	ARCaPE and ARCaPM cells are a model of epithelial-mesenchymal transition.	57
3.2	ARCaPM cells express high levels of ALPL mRNA and ALP activity compared to ARCaPE cells.	58
3.3	ALPL gene expression in prostate cancer cell lines	61
3.4	ARCaPM cells deposit inorganic mineral <i>in vitro</i> whereas ARCaPE cells do not mineralize.	63
3.5	Efficient knockdown of ALPL in ARCaPM cells alters their morphology.	65

3.6	Efficient knockdown of ALPL in ARCaPM cells reduces cell viability and increases cell death.	66
3.7	Knockdown of ALPL in ARCaPM cells decreases migration	67
3.8	Knockdown of ALPL in ARCaPM cells results in increased expression of epithelial marker E-cadherin	69
3.9	Knockdown of ALPL induces change in morphology, reduction in viability and increased cell death in PC3 cells	70
3.10	Pharmacological inhibition of ALP activity decreases viability and induces cell death in ARCaPM cells in a concentration dependent manner.	72
3.11	Snail knockdown in ARCaPM cells results in decreased ALP activity	74
3.12	ALPL expression is positively correlated with SNAI1 and negatively correlated with PTEN expression in human prostate cancer samples in the MSKCC dataset	76
4.1	Hypothesis	86
4.2	ATP induces a change to epithelial morphology and reduces viability in ARCaPM cells	89
4.3	Exposure to ATP results in increased expression of epithelial markers and decreased expression of mesenchymal markers in ARCaPM cells	91
4.4	Purinergic agonists other than ATP and ADP have little or no effect on cell viability in ARCaPE and ARCaPM cells	92
4.5	Automated quantitation of cell circularity using image acquisition and analysis on the BD Pathway	94
4.6	Non-specific blockade of purinergic receptors does not abrogate the effects of ATP in ARCaPM cells	95

4.7	The purinergic receptor P2X7 (P2RX7) is differentially expressed in a panel of prostate cancer cell lines	96
4.8	The ecto-nucleotidase apyrase does not rescue ARCaPM cells from ALPL knockdown	97
4.9	Effect of ATP treatment on alkaline phosphatase	98
5.1	Stable expression of miR-203 in ARCaPM cells reduces ALP and RUNX2 mRNA expression.	108
5.2	Stable expression of miR-203 in ARCaPM cells reduces mineralization and ALP activity.	109
5.3	miR clusters and families differentially expressed between ARCaPE and ARCaPM	113
5.4	Significantly differentially expressed microRNAs	114
5.5	Validation of miR-373 differential expression in ARCaPE vs. ARCaPM	115
5.6	MiR-373 overexpression induces mesenchymal-epithelial transition in ARCaPM cells.	117
5.7	ALP activity screen of microRNA mimic library before optimisation	119
5.8	ALP activity-based screening of a miR mimic library transfected in ARCaPM cells	120
5.9	Transfection of miR-199a-5p induces increased ALP activity in ARCaPM cells, but has no effect on viability	122
6.1	Summary	134
6.2	Combination of high-throughput screening techniques to identify microRNA regulators of EMT and migration in prostate cancer. . .	135

A.1	Levamisole induced a reduction in cell viability in 22Rv1 prostate cancer cells	136
A.2	Correlation of ALPL with CDH1 and ZEB1 gene expression in human prostate cancer samples	137
A.3	Apyrase degrades ATP in serum-free medium	138
A.4	Exogenous apyrase has no effect on levamisole-induced reduction in cell viability in ARCaPM cells	139
A.5	Exogenous porcine ALP has no effect on ARCaPE cell viability . .	140
A.6	An example of the optimisation performed for the development of the microRNA mimic library screen in 96-well format	141

List of Tables

1.1	TNM classification of prostate cancer	11
2.1	Primer sequences	47
3.1	Role of osteoblast-specific genes in prostate cancer	54
3.2	List of prostate cancer cells and their metastatic potential in animal models	60
5.1	RNA-seq: MicroRNAs with higher expression in ARCaPE	111
5.2	RNA-seq: MicroRNAs with higher expression in ARCaPM	112
B.1	Pyridoxine concentration in growth media	143

Abbreviations

ADP	- Adenosine diphosphate
ADT	- Androgen deprivation therapy
ALP	- Alkaline phosphatase
ALPL	- Alkaline phosphatase (bone/liver/kidney)
AMP	- Adenosine monophosphate
AR	- Androgen receptor
ARE	- Androgen response element
ATP	- Adenosine triphosphate
BMP	- Bone morphogenic protein
BPH	- Benign prostatic hyperplasia
Cat.	- Catalogue number
CRPC	- Castration resistant prostate cancer
CTC	- Circulating tumour cell
DHT	- Dihydrotestosterone
DNA	- Deoxyribonucleic acid
EGF	- Epidermal growth factor
EGFR	- EGF receptor
EMT	- Epithelial-mesenchymal transition
IGF	- Insulin-like growth factor
MET	- Mesenchymal-epithelial transition

miRNA - MicroRNA

MMP - Matrix metalloproteinase

mRNA - Messenger RNA

MSKCC - Memorial Sloan Kettering Cancer Center

PBS - Phosphate buffered saline

PCa - Prostate cancer

PIN - Prostatic intraepithelial neoplasia

PPADS - Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid

PSA - Prostate specific antigen

RANK - Receptor activator of NF- κ B

RANKL - RANK ligand

RNA - Ribonucleic acid

shRNA - short-hairpin RNA

siRNA - small interfering RNA

TGF β - Transforming Growth Factor β

TNAP - Tissue non-specific alkaline phosphatase

TRAP - Tartrate resistant acid phosphatase

uPA - Urokinase plasminogen activator

uPAR - uPA receptor

UTP - Uridine triphosphate

UTR - Untranslated region

VEGF - Vascular endothelial growth factor

Any fool can see the limits of seeing, but not even the wisest know the limits of knowing. Thus is ignorance rendered invisible, and are all Men made fools.

—R. Scott Bakker, *The White-Luck Warrior*

1

Introduction

1.1 Introduction

Prostate cancer (PCa) is the most common cancer in men in the western world. While primary prostate cancer is relatively non-lethal and indolent, resulting in a proportion of men with asymptomatic lesions, the cases that do progress to a metastatic disease, especially to the bone, often result in fatality. Up to 75% of all distant metastases in prostate cancer occur in the skeletal system and the mean 5-year survival of patients with bone metastases is estimated at around 25%. Skeletal metastasis in prostate cancer often occurs to the pelvis and vertebral column, although the involvement of long bones has also been reported and morbidity and mortality are due to secondary complications like pathological fractures in addition to increased tumour mass[2]. Hence, a detailed study of prostate cancer progression and bone metastasis is warranted, to identify the signalling pathways involved, which could lead to identification of novel therapeutic targets and diagnostic and prognostic markers.

1.1.1 History and epidemiology

The prostate gland was first described by Niccolo Massa of Venice[3] and was illustrated by Andreas Vesalius as early as 1543[4] (Fig. 1.1). Common diseases of the prostate include benign prostatic hypertrophy (BPH), prostatitis and prostate cancer. There is evidence of prostate cancer in ancient human samples. The oldest known case of metastatic prostate cancer was diagnosed in the well-preserved 2700-year old body of a Scythian king, showing osteosclerotic and osteolytic lesions in the skeleton in addition to detectable prostate specific antigen in the bone extracellular matrix[5]. The second earliest case of prostate cancer was discovered in an Egyptian mummy from the Ptolemaic period (c. 285–30 BC); computerised tomography of the mummy revealed multiple osteosclerotic lesions in the spine, suggestive of prostate cancer bone metastasis[6]. Initial methods of treatment for prostate cancer were mainly surgical. Radical prostatectomy was first performed by Hugh H. Young at the Johns Hopkins Hospital in 1905[7]. “Chemical castration”, using estrogen as an anti-androgen therapy, was first performed by Charles B. Huggins in 1941 and he received the Nobel Prize in Physiology or Medicine in the year 1966[8][9].

A



B

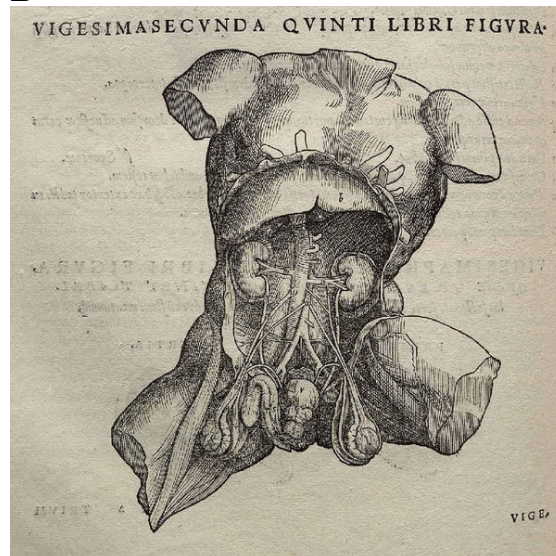
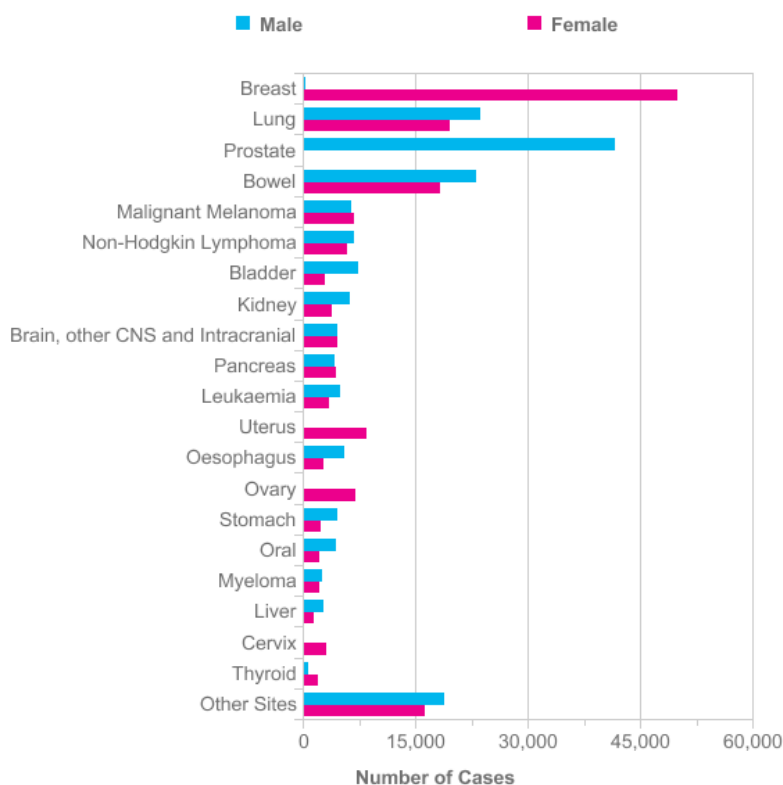


Figure 1.1: Andreas Vesalius (1514-1564) first illustrated the prostate gland (A) Chromolithograph of Andreas Vesalius at the dissection table, by Edouard Hamman, 1819-1888. (Wikimedia Commons, public domain) (B) Illustration of a dissected human body by Andreas Vesalius, depicting the male urogenital system, including the prostate and bladder[4]. (Wikimedia Commons, public domain)

Prostate cancer is the second most common among all cancers in terms of incidence and it is the third-most common cause of cancer-related deaths in the United Kingdom[10][11](Fig. 1.2). It accounts for 13% of all cancer cases (41,736 men in 2011) and 7% of all cancer deaths (10,837 men in 2011). In the year 2012, it was estimated that 417,000 new cases of prostate cancer were diagnosed in Europe and more than a million cases were identified worldwide. The incidence rate per 100,000 population in the UK is 104.7 and the mortality rate is 23.1[12]. The five-year survival rate for prostate cancer patients in England through the years 2005-2009 was 81.4%[12]. Survival rates in prostate cancer patients have improved significantly in the past 20-30 years due mainly to better screening methods, but also to some extent due to improved surgical outcomes. However, the survival rate in men with bone metastasis is much less, at only 47.4%[13]. The incidence of prostate cancer increases with age[14] and hence, in developed countries with ageing populations, like the UK, the disease burden is likely to increase in the future. This in turn leads to a heavy economic burden; prostate cancer in the European Union accounted for 7% of health care cost of all cancers, amounting to €8.43 billion in the year 2009[15].

The main and well established risk factors for prostate cancer are age, ethnicity and family history. Prostate cancer is mainly a disease affecting the elderly, with risk increasing with age. Incidence of prostate cancer is also known to vary in men of different ethnic origin. Men of African heritage were found to have a 3-fold higher risk of acquiring the disease compared to white males in the United States and the United Kingdom[16]. Heredity is also an important factor; an individual with a family history of prostate cancer in a first degree relative has twice the risk of being diagnosed with the disease, and the risk increases if more than one first degree relative is involved[17].

A



B

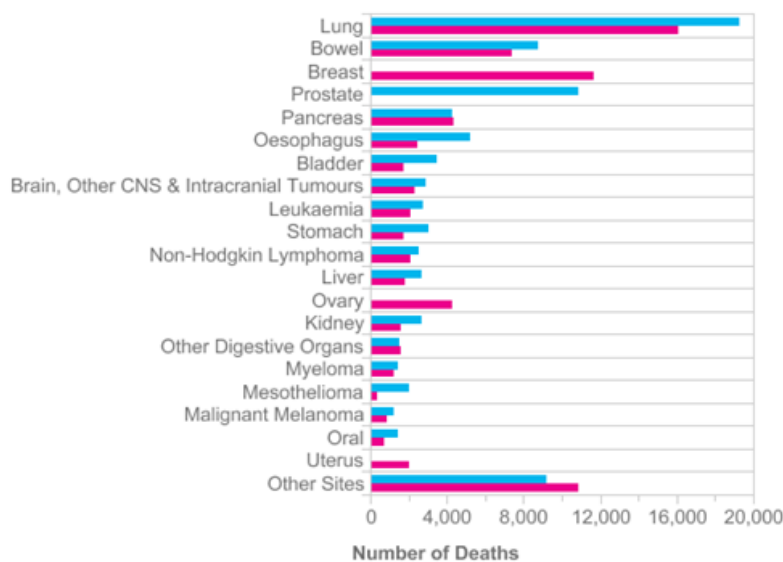


Figure 1.2: UK Statistics for 20 most common cancers (A) Incidence^[10] and (B) Mortality rates^[11] for the 20 most common cancers in the United Kingdom. (Adapted from Cancer Research UK)

1.1.2 Anatomy and physiology of the prostate gland

The prostate gland is a part of the male reproductive system located in the pelvic cavity. It is situated inferior to the urinary bladder, surrounding the proximal part of the urethra. The wider part of the prostate contiguous with the inferior wall of the urinary bladder is called the base of the prostate whereas the narrower part at the lower end is called the apex. It is separated from the surrounding organs and connective tissue by a band of fibrous tissue called the prostatic capsule, which is of importance in the clinical staging of prostate cancer[18]. The main function of the gland is production of seminal fluid, an alkaline secretion that constitutes 15-30% of semen. The alkalinity of the seminal fluid protects the spermatozoa from the acidic environment of the female reproductive tract, improving their survival. The prostate is the source of the proteins acid phosphatase and prostate specific antigen (PSA) which are present in the seminal fluid[19]. The prostate consists of 3 types of cells: glandular epithelial cells, myoepithelial cells and stromal cells[20]. The glandular epithelial cells are further classified into basal cells and luminal cells, which can be distinguished by their expression of specific cytokeratins, and the much rarer neuroendocrine cells. Basal cells express keratins 5 and 14 whereas keratins 8 and 18 are expressed by luminal cells[21]. Histologically, prostatic tissue is divided into various zones based on arrangement of glandular ducts and proximity to the urethra and ejaculatory ducts: the peripheral zone consisting of nearly 70% of the total prostate, the central zone constituting 25% of the prostate gland and surrounding the ejaculatory ducts (and encircled by the peripheral zone), the transitional zone that consists of fibromuscular tissue and the periurethral zone surrounding the urethra[22][23].

1.1.3 Prostate tumourigenesis

Almost all prostate carcinomas originate in the peripheral zone whereas BPH is primarily due to continued growth of the transitional zone with increasing age. There is no clear consensus on the cell of origin of prostate cancer. Both luminal and basal cells have been proposed in this role and considerable controversy exists in the field regarding this[24][25][26]. Regardless of the cell type, prostate tumours start as intraepithelial neoplasias (PIN), which are confined to the glandular epithelium limited by the basement membrane. Subsequently, the tumour cells escape through the basement membrane and form larger tumours in the prostate mesenchyme. Early stages of carcinoma that is confined to the prostate are often asymptomatic or the patient may present with difficulty in urination due to obstruction of the prostatic urethra(Fig. 1.3A). Later, the tumour cells invade through the prostatic capsule into the surrounding tissues. Tumour cells also spread to lymph nodes in the pelvic region through the lymphatic drainage and to distant organs through the blood supply[27](Fig. 1.3B).

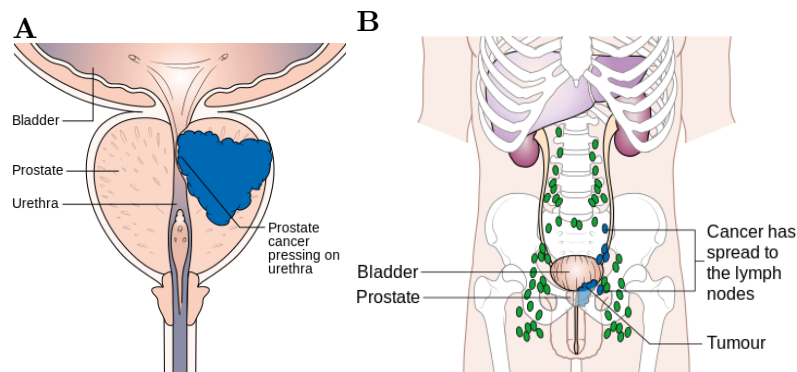


Figure 1.3: Clinical features of prostate cancer (A) Illustration of the prostate gland, with a tumour compressing the urethra. (Cancer Research UK / Wikimedia Commons, CC BY-SA 4.0) (B) Malignant tumours arising in the prostate gland can spread to the regional lymph nodes through the lymphatic supply. (Cancer Research UK / Wikimedia Commons, CC BY-SA 4.0)

1.1.4 Clinical features

Diagnosis

Digital Rectal Examination (DRE)

Palpation of the prostate gland by rectal examination is an important diagnostic technique to identify prostate cancer. Identification of an abnormal mass or hardness of the prostate by DRE is an indication for a biopsy.

Prostate Specific Antigen (PSA)

PSA is a serine protease normally expressed by the glandular epithelial cells of the prostate gland. Its level in serum is increased in prostate cancer, but this is not a specific marker of malignant disease as it is also increased in benign conditions like BPH and prostatitis. Despite this, it has been the mainstay of prostate cancer screening and is thought to have helped in early diagnosis and treatment since the development of a serum test in 1980[28]. However, there is considerable controversy over its use, with concerns that over-diagnosis and over-treatment may in fact be harmful to the patient when used as an indiscriminate screening method[29]. Randomized controlled trials have shown that PSA screening provides no overall survival benefit[17].

Prostate biopsy

A biopsy allows histological examination of the prostate gland and is necessary for a diagnosis of prostate cancer. A decision to perform a biopsy is based on DRE and PSA levels[17].

Diagnosis of bone metastasis

The presence of bone metastases in prostate cancer is determined by X-ray imaging and radionuclide bone scintigraphy (bone scan). X-ray images may show abnormal shadows or opacities in the axial skeleton, suggestive of osteolytic and osteoblastic bone metastases(Fig. 1.4A). A bone scan involves administration of radioactive

technetium which has a tendency to localise in bones, particularly in areas of high bone turnover. Metastatic sites show up as dark spots in the bone scan due to higher radioactivity[30](Fig. 1.4B).

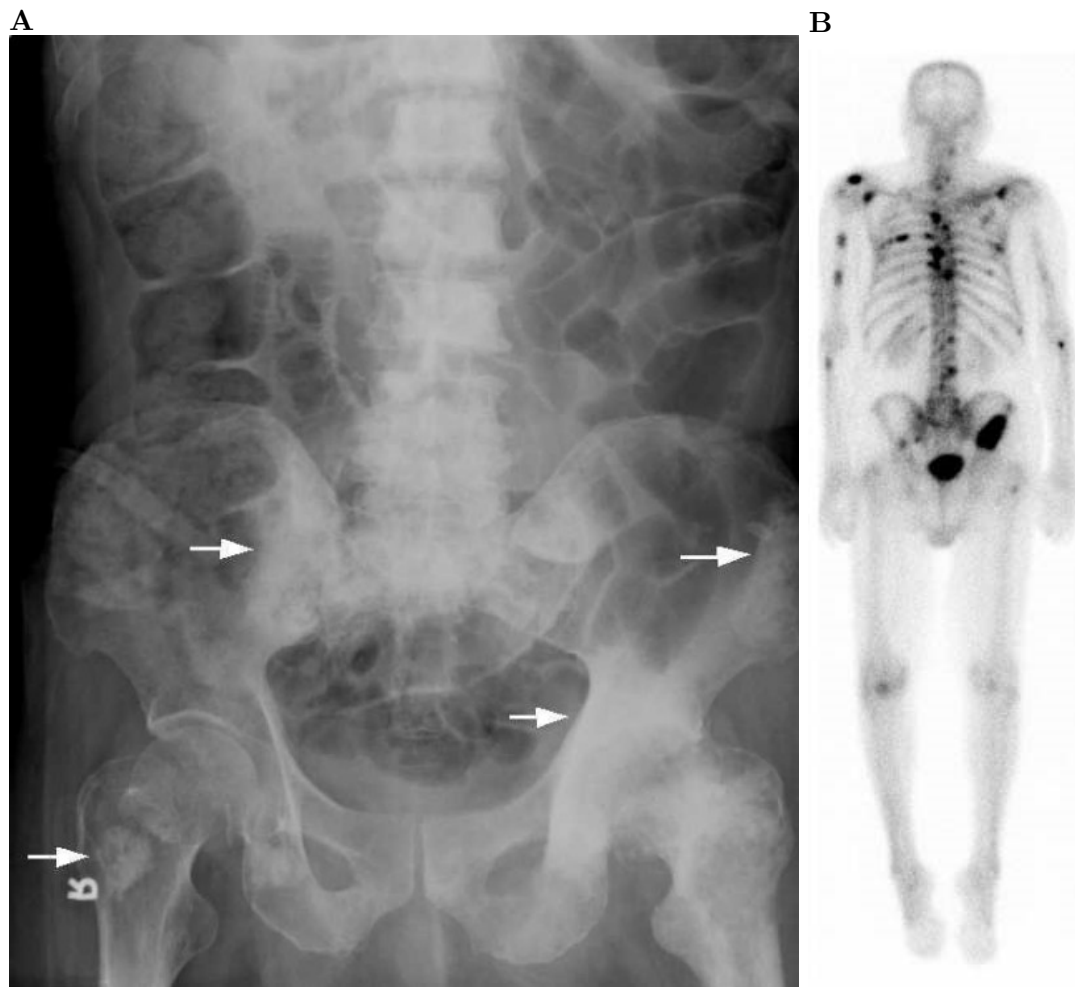


Figure 1.4: Bone metastasis in prostate cancer (A) Radiograph of a 70 year-old prostate cancer patient, with white arrows pointing to areas of osteoblastic bone metastasis[31]. (Open Access under CC-BY-2.0) (B) Radionuclide scintigraphy of a patient with prostate cancer bone metastases. (Wikimedia Commons/radswiki.net, CC BY-SA 3.0)

Clinical staging

Based on the extent and spread of the tumour, prostate cancer is classified using the TNM system[17]. Broadly, organ confined tumour is considered less aggressive whereas extension of the tumour beyond the prostatic capsule, involvement of surrounding tissues, lymph nodes and distant metastasis being considered as signs of increasing severity and poorer prognosis(Table 1.1).

Gleason grade and score

Gleason grade is a system of prostate tumours based on their pathological appearance. Features such as conservation of normal glandular appearance, tumour shape, invasion into surrounding stroma and shape of the tumour cells are used to assign a grade. 5 grade patterns have been described and a primary grade ranging from 1-5 is assigned based on the most common pattern seen in histology. A secondary grade, also ranging from 1-5 is assigned to the second most common pattern observed. The primary and secondary grades are then added to derive a number ranging from 1-10, called the Gleason score. The Gleason grading system and the Gleason score are considered to be a very important indicator of prognosis in prostate cancer[32].

Table 1.1: TNM classification of prostate cancer [17]

T - Primary tumour
TX Primary tumour cannot be assessed
T0 No evidence of primary tumour
T1 Clinically inapparent tumour not palpable or visible by imaging
T1a Tumour incidental histological finding in 5% or less of tissue resected
T1b Tumour incidental histological finding in more than 5% of tissue resected
T1c Tumour identified by needle biopsy (e.g. because of elevated prostate-specific antigen [PSA] level)
T2 Tumour confined within the prostate
T2a Tumour involves one half of one lobe or less
T2b Tumour involves more than half of one lobe, but not both lobes
T2c Tumour involves both lobes
T3 Tumour extends through the prostatic capsule
T3a Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement
T3b Tumour invades seminal vesicle(s)
T4 Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall
N - Regional lymph nodes
NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Regional lymph node metastasis
M - Distant metastasis
MX Distant metastasis cannot be assessed
M0 No distant metastasis
M1 Distant metastasis
M1a Non-regional lymph node(s)
M1b Bone(s)
M1c Other site(s)

1.1.5 Treatment and remission

Men diagnosed with low risk prostate cancer may be kept under active surveillance or “watchful waiting” with no surgical intervention. This is because prostate cancer is an indolent disease and is often asymptomatic in early stages; it has been suggested that unnecessary surgical procedures and their associated complications can be avoided in a large number of such low risk cases. In patients with low to intermediate risk prostate cancer where the tumour is confined to the prostate gland, the primary modality of treatment is radical prostatectomy which involves the surgical resection of the entire organ. Regional lymph nodes may also be removed as part of radical prostatectomy. In patients with intermediate to high risk prostate cancer, in addition to radical prostatectomy, adjuvant external-beam radiation therapy following surgery is deemed beneficial[33].

In patients with metastatic prostate cancer or in cases of relapse following surgery and radiation therapy, hormonal therapy is the treatment of choice. Also known as androgen deprivation therapy (ADT), hormonal therapy in prostate cancer involves the administration of luteinising hormone-releasing hormone (LHRH) agonists and nonsteroidal antiandrogens. As the disease progresses to castrate or castration resistant prostate cancer (CRPC), the tumour growth is resistant to hormonal therapy. At this stage, chemotherapeutic agents like Sipuleucel-T, Abiraterone with prednisone and Docetaxel are recommended first-line treatment. When the disease progresses to bone metastasis, the treatment is largely palliative, to prevent bone disease and consequent pathological fractures. Zoledronic acid, a bisphosphonate that reduces bone pain and destruction, Denosumab, an antibody against receptor activator of NF- κ B ligand that reduces bone destruction, and radium-223 which prevents excessive bone formation, are the recommended treatments of choice at this stage[34].

1.1.6 Metastasis in cancer

Metastasis is a process by which cancer cells from the primary tumour spread to distant sites and establish secondary tumours. Along with invasion, it was proposed as one of the original 6 hallmarks of cancer[35]. It is a multi-step process that involves escape of tumour cells from the organ of origin, entry into blood or lymphatic vessels (intravasation), traversal through circulation (circulating tumour cells), escape from blood vessels (extravasation), and colonisation at secondary sites[36](Fig. 1.5).

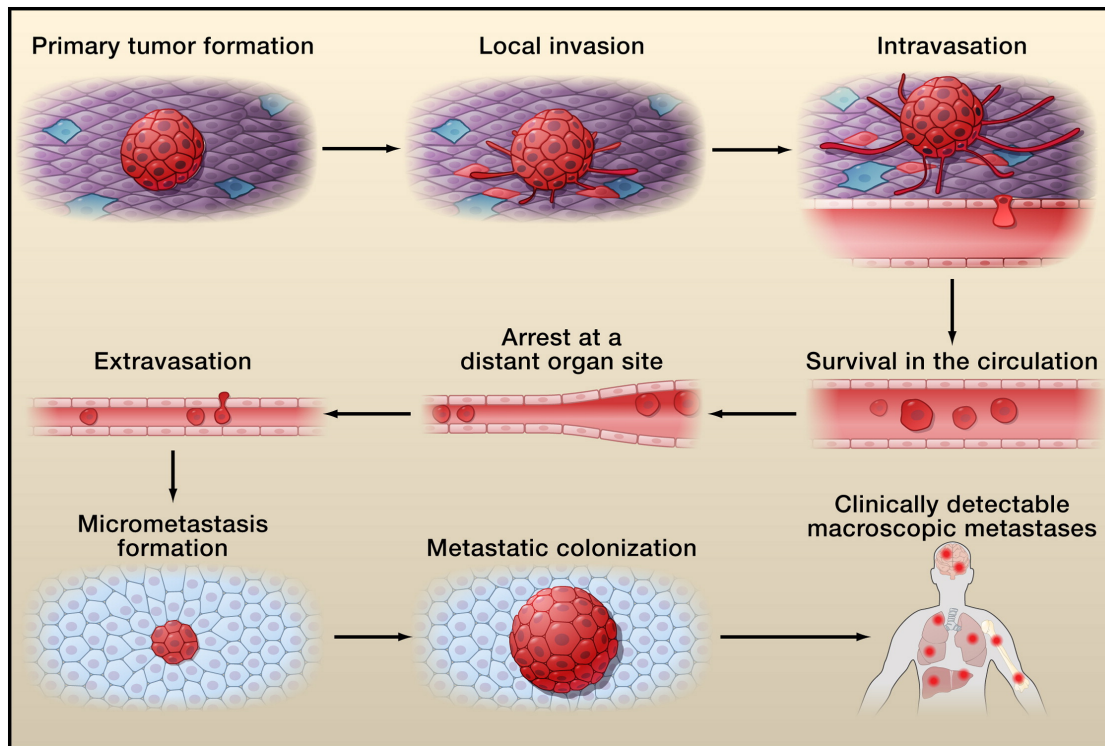


Figure 1.5: Steps involved in metastasis of cancer cells to distant sites [36] (reproduced with permission from Elsevier)

1.1.7 Bone metastasis in prostate cancer

Prostate cancer cells have a predilection to metastasize to bone, although they are known to less frequently metastasize to other organs like liver, lungs and adrenal glands. Stephen Paget proposed the seed and soil hypothesis in 1889[37] to explain the predisposition of breast cancer cells to metastasize to bone. This was revived in 1980 by Hart and Fidler[38] and has been used to explain organ-specific metastasis in other cancers. The mechanisms underlying the propensity of prostate cancer cells to metastasize to bone and the development of the associated bone disease are poorly understood. Bone remodelling involves destruction of existing bone matrix by osteoclasts and formation of new bone by osteoblasts. This process is regulated by a crosstalk between osteoblasts and osteoclasts called “coupling”, which requires a tightly regulated turnover of the bone matrix[39]. Prostate cells are known to interact with both osteoblasts and osteoclasts and thereby upset the critical balance required for bone-remodelling, resulting in the destruction or formation of bone at undesirable locations. Although bone metastasis in prostate cancer are conventionally thought to be predominantly osteoblastic, recent evidence suggests that osteolysis is also significantly involved and may more accurately be termed as mixed (osteoblastic + osteolytic) lesions. This is in contrast to the bone disease in multiple myeloma and breast cancer; bone metastases in both these malignancies produce purely osteolytic lesions.

1.2 The bone microenvironment

The skeletal system predominantly consists of bone, which is made up of the bone matrix and bone cells. The majority (approximately 98%) of the bone matrix is inorganic and composed of hydroxyapatite $Ca_5(PO_4)^3(OH)$ whereas structural proteins (mainly collagen and osteocalcin) constitute the organic matrix. New bone is

formed by osteoblasts whereas bone is degraded by osteoclasts. Various signalling pathways interconnect osteoblasts and osteoclasts, maintaining a fine balance between bone formation and destruction (coupling, described above)[40]. Changes in the local concentration of endocrine factors, inflammation or factors secreted by tumour cells may upset this balance, leading to excessive and/or abnormal bone formation and destruction[41]. Osteocytes are ensconced in the mineralized bone matrix and are involved in mechanosensing and communicate with each other through long cytoplasmic processes[42]. Mesenchymal stem cells are present in the bone microenvironment and can differentiate to osteoblasts or adipocytes[43]. In addition to these, the bone marrow is home to a number of haematopoietic and immune cells.

1.2.1 Osteoblasts

Osteoblasts are the main source of alkaline phosphatase in bone and also express a number of structural proteins. They are thus the principal contributors of the bone matrix[44]. Mesenchymal stem cells in bone initially differentiate into bipotential precursor cells that can either further mature to osteoblasts or adipocytes[45].

Runx2

Runt-related transcription factor 1 (Runx2) also known as core-binding factor α (CBFA) is a transcription factor that induces the expression of a number of skeletal genes, including tissue non-specific alkaline phosphatase, collagen, osteocalcin, RANKL and osteopontin[46][47], and is a crucial mediator in the differentiation of mesenchymal stem cells to osteoblasts[44].

Alkaline phosphatase (bone/liver/kidney)

Alkaline phosphatase (bone/liver/kidney) (ALPL), also known as tissue non-specific alkaline phosphatase (TNAP) is a membrane-bound protein with an extracellular

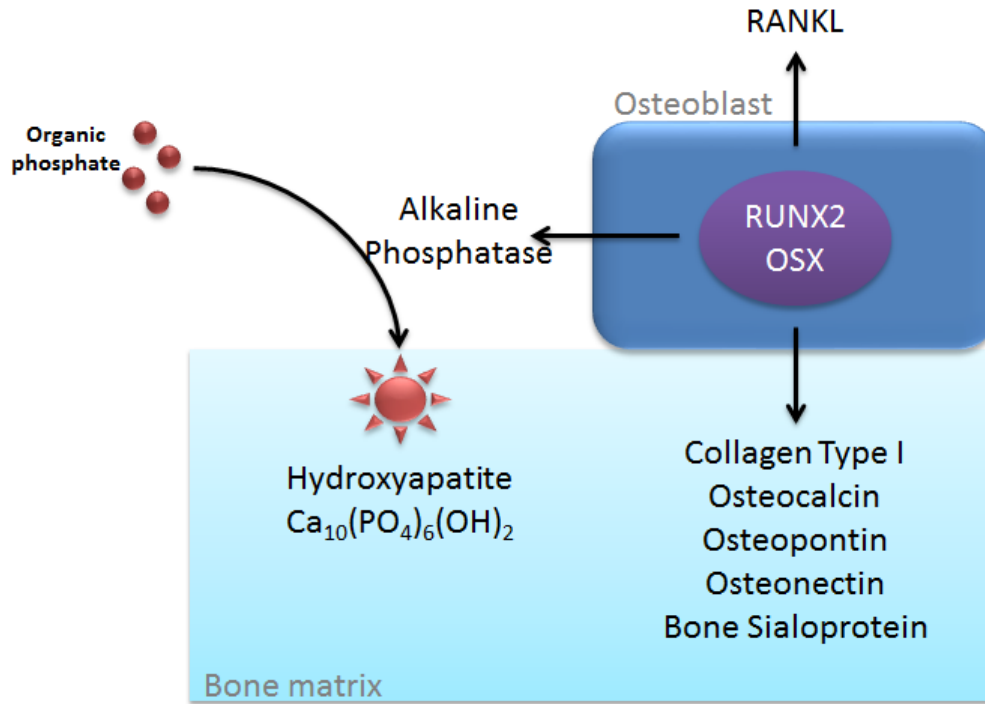


Figure 1.6: Factors expressed by an osteoblast The osteoblast, which is responsible for the deposition of the organic and inorganic part of the bone matrix, expresses many structural proteins like collagen, osteocalcin and bone sialoprotein, as well as the enzyme alkaline phosphatase.

catalytic domain. It is abundantly expressed in bone (osteoblasts), liver, kidneys, lungs, blood (leukocytes) and to some extent in the brain and spinal cord[48]. It cleaves phosphate groups from organic substrates like ATP and pyridoxal phosphate; in the bone microenvironment, the free phosphate reacts with calcium to form hydroxyapatite. Homozygous knockout of alkaline phosphatase in mice results in decreased growth and seizures. The neurological effects in ALPL knockout mice are due to the accumulation of pyridoxal phosphate, which is normally dephosphorylated by alkaline phosphatase[49]. Alkaline phosphatase plays a pivotal role in bone mineralization; knockout of ALPL (Akp2), the mouse homologue of the human ALPL gene, results in a significant decrease in mineralization[50][51].

Reconstitution of vitamin B6 in ALPL knockout mice rescues the neurological phenotype but not the defect in mineralization, suggesting that both the phenotypes are due to distinct functions of ALPL[52]. There is also strong *in vitro* evidence demonstrating that alkaline phosphatase is necessary and sufficient to induce mineralization in cultured osteoblasts[53] and it is widely considered an early marker of osteoblast differentiation. However, the role of alkaline phosphatase in other tissues is largely unknown. Deficiency of functional alkaline phosphatase in humans, known as hypophosphatasia, is associated with impaired mineral deposition in the bones[54]. Other isoforms of ALP are produced by three additional genes: ALPI (intestinal or enteric), ALPP (placental) and ALPPL2 (placental-like)[55]. Unless otherwise specified, alkaline phosphatase is used in this thesis to refer to alkaline phosphatase (bone/liver/kidney) (ALPL). While alkaline phosphatases in hepatocytes, osteoblasts and renal cells (in the proximal convoluted tubules) are all produced by the same gene ALPL, the resultant protein isoforms are thought to be slightly different from each other in their structure due to post-translational modifications like glycosylation[56].

Collagen 1 α

Collagen 1 α , also known as type I Collagen is the most abundant matrix protein in bone and is an early marker of osteoblast differentiation. It is also highly expressed in other connective tissues, providing structural integrity and strength. The triple-helical structure of Collagen, combined with its tendency to organise into fibres makes it important in its structural role[57].

RANKL

The tumour necrosis factor ligand superfamily member 11 (TNFSF11), also known as receptor activator of NF- κ B ligand (RANKL) is a primarily secreted protein that

binds to tumour necrosis factor receptor superfamily member 11A (TNFRSF11A), also known as receptor activator of NF- κ B (RANK). This initiates a signalling cascade mediated by NF- κ B, which is a crucial mechanism in the differentiation of macrophage precursor cells to osteoclasts. RANKL is normally expressed by osteoblasts, in addition to other tissues like thymus, lungs and lymph nodes[58].

1.2.2 Osteoclasts

Haematopoietic cells of the macrophage lineage differentiate into osteoclasts in the bone microenvironment. Mature osteoclasts are multi-nucleated giant cells that are formed by fusion of macrophage precursor cells[59]. Their primary function is bone destruction, which is achieved by the secretion of tartrate resistant acid phosphatase (TRAP) and the proteolytic enzyme Cathepsin K. These enzymes help in degrading both the inorganic and organic components of the bone matrix. Mature osteoclasts seal off a portion of the bone surface and pump H^+ ions that maintain an acidic environment for optimal function of TRAP[60]. Osteoclast activity may be deduced by the presence of resorption pits (areas of degraded bone matrix) and staining for TRAP through immunohistochemistry[61]. RANK receptors present on the osteoclast precursor cell surface bind to RANKL, which results in osteoclastogenesis. The secreted protein osteoprotegerin also binds to RANKL and is considered a decoy receptor, preventing RANKL binding to RANK.[62].

1.2.3 Interaction of prostate cancer cells with the bone micro-environment

Prostate cancer cells, when they reach bone, establish a cross-talk with cells in the bone microenvironment. The major cell types of the bone and bone marrow are osteoblasts, osteoclasts, and cells of the haematopoietic niche. RANKL

is an important factor in the formation of osteolytic lesions in prostate cancer bone metastasis. Prostate cancer cells express elevated levels of RANKL, which induces osteoclast differentiation, thus increasing the destruction of bone[63][64]. The mechanisms involved in the formation of osteoblastic lesions is less well understood. Endothelin-1 (ET1) is causally associated with osteoblastic bone lesions in models of breast cancer metastasis[65]. The interaction of prostate cancer cells with the bone matrix is also thought to result in the induction of osteoblast differentiation and proliferation. Precursor or latent forms of several growth factors, including Transforming Growth Factor β (TGF β), Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF) are thought to be impregnated in the bone matrix, and released during bone turnover triggered by the tumour cells[41]. Proteases secreted by prostate cancer cells are thought to release these growth factor precursors from the bone matrix; they are subsequently converted by the proteases present in the bone microenvironment to their active forms, thereby stimulating the function of osteoblasts. These growth factors are also thought to act on the prostate cancer cells, enhancing their proliferation through a feedback mechanism[66].

1.3 Factors with a role in prostate cancer progression and bone metastasis

1.3.1 Androgen receptor

The androgen testosterone is produced by the Leydig cells of the testes[67]. Testosterone enters the prostate cells by passive diffusion and is converted by the enzyme 5- α -reductase to dihydrotestosterone (DHT). Androgen receptor (AR) is an intracellular steroid receptor normally present in the cytoplasm when in an inactive state. It is maintained by chaperone-binding in an inactive conformation; upon

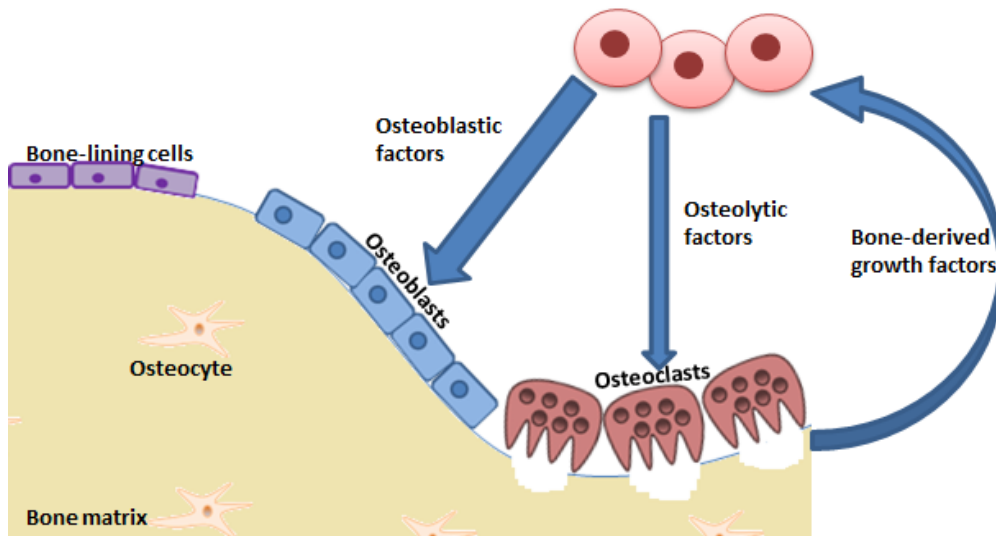


Figure 1.7: Prostate cancer cells cross-talk with bone cells Metastatic prostate cancer cells that reach the bone microenvironment establish a cross-talk with the cells there. Factors expressed by the tumour cells increases differentiation and proliferation of osteoblasts and osteoclasts, resulting in both increased abnormal bone formation and destruction.

binding to DHT, AR changes into its active conformation and is separated from its chaperones. Once in the active conformation, the nuclear localisation signal is exposed, leading to the translocation of AR into the nucleus[68]. Active AR binds to the Androgen Response Element (ARE) motifs in the promoter regions of its target genes; the ARE motif consists of the consensus sequence AGAACA_nnnT-GTTCT (AGAACA arranged as an inverted repeat, separated by 3 base pairs)[69]. In prostate cancer cells, AR transactivates the expression of a number of genes responsible for cell proliferation[70]. AR signalling has a cross-talk with many other proliferative and growth factor signalling pathways. AR also has a number of co-activators that help in its stability and transactivation, including the transcription factors β -catenin, Smads2/3[71] and members of the PIAS (protein inhibitor of activated signal transducer and activator of transcription (STAT)) family (PIAS1

and PIASx β)[72].

1.3.2 PTEN

PTEN is a tumour suppressor deregulated in many cancers and is mutated in up to 20% of prostate tumours. PTEN inhibits PI3KAKT signalling by catalysing the dephosphorylation of phosphatidylinositol (3, 4, 5) triphosphate (PIP3) to phosphatidylinositol (4, 5) diphosphate (PIP2), resulting in the inhibition of cell proliferation and survival and promotion of apoptosis. Overexpression of PTEN in prostate cancer cells leads to increased apoptosis and cell cycle arrest[73] and its loss is associated with poorer prognosis[74]. Homozygous knockout of PTEN alone in the mouse prostate replicates all the stages of prostate cancer progression, from prostatic intra-epithelial neoplasia (PIN) to invasion and bone metastasis and the development of castration resistant prostate cancer (CRPC)[75]. PTEN loss is therefore considered a crucial step in the development of advanced prostate cancer.

1.3.3 TNFSF11/RANKL

RANKL, normally expressed by osteoblasts, is frequently overexpressed in prostate cancer bone metastases[76] and plays a significant role in the consequent bone disease by promoting osteoclastogenesis and bone destruction[77]. High NF- κ B activity is associated with increased RANKL expression in PC3 and LNCaP cells and results in greater tumour growth in the bone microenvironment[78]. RANKL is also a mesenchymal marker and stimulation by TGF- β and EGF results in increased expression of RANKL[79][80].

1.3.4 Endothelin-1

Endothelin-1 (ET-1) is a secreted 21 amino acid peptide produced by vascular endothelial cells and induces vasoconstriction[81]. The serum expression of ET-1

is elevated in metastatic prostate cancer patients and increases cell proliferation *in vitro* in PC3, Du145 and LNCaP cells[82]. It acts by binding to the Endothelin A and Endothelin B receptors, which are expressed on the osteoblast cell surface. Activation of the receptors results in osteoblast proliferation, leading to excess bone formation. Endothelin-1 secreted by ZR-75-1 breast cancer cells is thought to act on osteoblasts in this way, resulting in osteoblastic bone metastases, and pharmacological inhibition of Endothelin A receptor in mice results in a reduction of osteoblastic bone lesions[65].

1.3.5 Runx2

In addition to the crucial role of Runx2 in the expression of osteoblast markers (discussed above), it also transactivates the expression of cancer-related genes, including MMP-9, MMP-13 and MDR-1. Runx2 is frequently overexpressed in prostate cancer bone metastases and is associated with the tumour aggressiveness[83]. Non-metastatic LNCaP cells express minimal levels of Runx2 whereas the highly metastatic PC3 cells express abundant Runx2. Knockdown of Runx2 in PC3 cells results in decreased migration and invasion *in vitro* and diminished tumour formation and bone lesions in an intra-tibial mouse model[83].

1.3.6 Parathyroid Hormone-related Peptide (PTHrP)

PTHrP is an endocrine hormone that regulates endochondral bone formation and promotes osteoclastic activity. It is expressed in primary as well as bone metastatic prostate cancer[84]. Overexpression of PTHrP in non-invasive Du-145 cells confers a bone metastatic ability, resulting in the formation of osteolytic bone lesions[85] and protects LNCaP cells from drug-induced apoptosis *in vitro*[86]. Overexpression of PTHrP in prostate cancer cells in another mouse bone metastasis model also increases formation of osteoblastic and osteolytic lesions[87].

1.3.7 Dickkopf-1 (DKK1)

DKK1 is secreted protein that antagonises the Wnt signalling pathway by binding to LRP6. In normal bone, it regulates osteoblast differentiation; loss of DKK1 function results in excessive bone formation[88]. DKK1 expression is significantly elevated in prostate cancer bone metastasis and is known to play an important role in the development of osteolytic bone disease[89][90].

1.3.8 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are enzymes that degrade structural proteins like collagen and fibrin and are important in cell migration during embryonic development. MMPs, particularly MMP-2 and MMP-9, are frequently upregulated in various cancers including prostate tumours. Increased MMP expression allows the prostate cancer cells to degrade basement membranes and connective tissue during their migration and colonisation of the bone microenvironment, a large part of which is composed of an organic matrix. Overexpression of MMP-9 increases the invasiveness of LNCaP cells[91] and MMP-9 regulates the expression of angiogenic factors in PC3 cells[92]. MMP-7 is overexpressed at the tumour-bone interface in a mouse model of prostate cancer and ablation of MMP-7 in the host mouse prevents formation of osteolytic bone lesions[93].

1.3.9 uPA and uPAR

Urokinase plasminogen activator (uPA) and its cognate receptor (uPAR) are known to be important mediators of prostate cancer invasion and metastasis. uPA is a serine protease that is secreted extracellularly in a pro-enzyme form with basal activity. Its activity is increased many-fold when it binds to uPAR, leading to extracellular or intracellular consequences. Outside the cell, another serine protease plasminogen is activated due to cleavage by active uPA, giving rise to plasmin.

Plasmin is crucial in further activation of other enzymes in the tumour microenvironment like elastase and MMPs. Together, uPA and other proteases activated by it can cleave extracellular matrix proteins like laminin, fibronectin, vitronectin and collagen in the basement membrane and tumour microenvironment; this is considered an essential step in prostate cancer progression during the escape of tumour cells from the basement membrane of the glandular epithelium, invasion through prostatic stroma, intravasation into blood vessels and extravasation at distant sites. These proteases are also thought to be crucial in the colonisation of bone by the tumours as they release and activate many latent growth factors that are impregnated in the bone matrix[94]. In addition, activation of uPAR by uPA also activates intracellular signalling pathways, including the Jak/Stat and JNK/Jun pathways; knockdown of uPA and uPAR alone or in combination decreased invasive potential and proliferation and increased apoptosis in PC3 cells[95]. uPA and uPAR are elevated in the serum of prostate cancer patients compared to men with BPH and are decreased after radical prostatectomy[96]. Increased uPA and uPAR expression in tissues is associated with prostate cancer progression, grade and disease outcome[97]. Further, uPA and uPAR expression was also found to be elevated in the cancer-associated stroma of higher grade prostate cancer compared to that associated with lower grade tumours[98].

1.3.10 Growth factors

Epidermal Growth Factor Receptor (EGFR) signalling

EGFR (HER2, ERBB1) is a cell surface receptor belonging to the ERBB family of proteins. It is activated by the growth factor ligands EGF and TGF- β ; the receptor exists as a monomer in an inactivated state and homodimerizes following activation. This triggers tyrosine kinase activity in its intracellular domain, leading to the activation of a number of signalling pathways. EGFR activation triggers

the MAPK, AKT, JNK and Wnt pathways among others, all of which typically lead to increased cell proliferation, migration and invasion. EGFR expression is elevated in metastatic prostate cancer and is associated with gleason grade and PSA levels. Patients with high EGFR levels are also more prone to relapse of prostate cancer following radical prostatectomy[99]. Missense mutations in prostate cancer can lead to constitutive activation of EGFR signalling[100]. EGF stimulated the proliferation of PC3, Du145 and LNCaP cells by triggering tyrosine kinase activity[101]. Pharmacological inhibition of EGFR signalling induced apoptosis in prostate cancer cells by inhibiting NF- κ B signalling[102].

ERBB2 (HER2/Neu) is another member of the ERBB protein family; while it has no known natural ligands, it forms heterodimers with other members of the family like EGFR and ERBB3, activating growth signalling. ERBB2 signalling activates androgen receptor-mediated transcriptional activity in prostate cancer cells and pharmacological inhibition of ERBB2 resulted in impaired development of the prostate and seminal vesicles in mice[103].

Insulin-like Growth Factor (IGF) signalling

IGF1 receptor (IGF1R) is a cell surface protein in the Insulin receptor family and is activated by the growth factor ligand IGF1. Upon activation, downstream signalling cascades mediated mainly by Ras/MEK/ERK and AKT are triggered, leading to cell proliferative gene transcription[104]. Increased serum levels of IGF1 are associated with elevated risk of prostate cancer[105]. Increased IGF1R in prostate cancer patients is also associated with worse prognosis[106]. IGF1R expression is increased in malignant primary prostate cancer cells compared to benign epithelium and its expression is increased in metastatic prostate cancer[107]. IGF1R activation promotes growth in prostate cancer cells through the activation of various signalling pathways including stabilisation of $\alpha_5\beta_1$ integrin[108],

activation of β -catenin[109] and activation of STAT3-Twist1 pathways[109]. It is thought that IGF1R/AKT signalling compensates for the lack of androgen in androgen-independent prostate cancer cells by transactivating the androgen receptor[110]. The microRNA let-7a inhibits growth and induces apoptosis in PC3 cells by directly targeting the IGF1R 3' UTR[111] and is down-regulated in castration resistant prostate cancer[112]. Further, inhibition of IGF1R sensitises prostate cancer cells to chemotherapeutic agents and ionising radiation[113].

Vascular Endothelial Growth Factor (VEGF) signalling

The growth factors VEGFA, VEGFB, VEGFC, and VEGFD belong to the VEGF family and are expressed in a paracrine manner. VEGFA is the most well studied among these and is found as a number of variants due to alternative splicing. Each of these variants has different activity and binding capacity to the cognate receptors. VEGFA binds to VEGFR1 (FLT1), VEGFR2 (FLK1) and VEGFR3 (FLT4), all of which are receptors with intrinsic tyrosine kinase activity that is induced following ligand binding[114]. VEGFA is normally expressed by endothelial cells and stimulates neovascularisation and angiogenesis in physiological conditions. However, a number of cancer cells express VEGFA; rapid tumour growth results in insufficient supply of oxygen and nutrients and tumour cells circumvent this by increased angiogenesis. VEGF is known to be expressed in prostate cancer[115][116] although its expression in BPH is debated[117]. Inhibition of VEGF using neutralizing antibodies results in decreased tumour growth in a mouse model of prostate cancer[118] and it has also been implicated to have a role in the development of osteoblastic bone metastasis[119].

Bone Morphogenic Protein (BMP) signalling

Bone morphogenic proteins are highly evolutionarily conserved growth factors belonging to the transforming growth factor- β superfamily. They bind to BMP receptors and activin receptors, inducing a signalling cascade involving Smad1/5 leading to transactivation of mainly regulating skeletal morphogenesis, limb development and patterning[120]. BMP-6 was found to be overexpressed in malignant prostate cancer samples compared to their benign controls and is especially elevated in metastatic samples[121]. BMP-7 is expressed in many tissues including bone marrow and the prostate gland and is a physiological inhibitor of EMT. It has been shown to inhibit branching morphogenesis in the development of the mouse prostate by interfering with the Notch signalling pathway[122]. BMP-7 expression is significantly reduced in prostate cancer samples compared to the normal prostate and is also decreased in cell lines with increased metastatic potential. Treatment of prostate cancer cells *in vitro* with BMP-7 protein resulted in decreased cell growth and increased E-cadherin expression. In vivo, administration of BMP-7 decreased tumour growth in bone, but not orthotopic tumours in the prostate[123]. However, these findings appear to be contradicted by a report that BMP-7 induces EMT in PC3 cells[124]. Morrissey et al. have shown that BMP-7 expression is decreased in primary prostate cancer compared to the normal prostate, but increased expression in CRPC compared to androgen-responsive prostate cancer. They have therefore suggested that the role of BMP-7 in prostate cancer varies based on androgen responsiveness and the tumour microenvironment[125].

1.4 Epithelial-Mesenchymal Transition

Epithelial cells are characterised by a rounded morphology with high cell-cell adhesion *in vitro*, decreased migration and invasion both *in vitro* and *in vivo*,

and expression of epithelial markers like E-cadherin[126] and cytokeratins 14, 18, 19[127]. In contrast, cells with a mesenchymal phenotype are characterized by a spindle shape and decreased surface dependence *in vitro*, invasiveness both *in vitro* and *in vivo*, and expression of mesenchymal markers like ZEB1[128], ZEB2[129], Snail[130], Slug, Twist, Vimentin and Fibronectin[127]. Cells can transform from an epithelial to mesenchymal phenotype due to various genetic, epigenetic or environmental factors. This phenomenon is termed epithelial-mesenchymal transition (EMT). The converse process of transformation from a mesenchymal to an epithelial phenotype is termed as mesenchymal-epithelial transition (MET). EMT may occur in physiological or pathological processes and is classified into type 1 (embryogenesis and development), type 2 (wound healing) and type 3 (cancer) depending on the context[127]. Epithelial cells exhibit a distinct basement membrane-lumen polarity and a high degree of cell-cell adhesion. This is maintained by the expression of tight and adherens junctions at the cell surface. EMT involves dramatic changes in cell polarity, with the destruction of cell-cell junctions and reorganisation of cytoskeleton[131]. Actin stress fibres are formed during the transition, which help in the formation of cytoplasmic extensions called invadopodia. These cytoskeletal changes increase cellular contractility and motility allowing the cells to squeeze through narrow spaces. Invadopodia also secrete enzymes such as matrix metalloproteinases which can cleave extracellular matrix proteins like collagen and fibronectin present in connective tissue and basement membrane[132][133]. While EMT is important during development and wound healing, metastatic cancer cells can hijack these physiological pathways resulting in increased migration and invasion. EMT has been linked to cancer stemness[134], with increased EMT resulting in de-differentiation. Enzymes secreted by mesenchymal cells also help them to break down the basement membrane during EMT[135] and this is thought to help cancer cells escape the primary tumour site into nearby blood vessels. Fur-

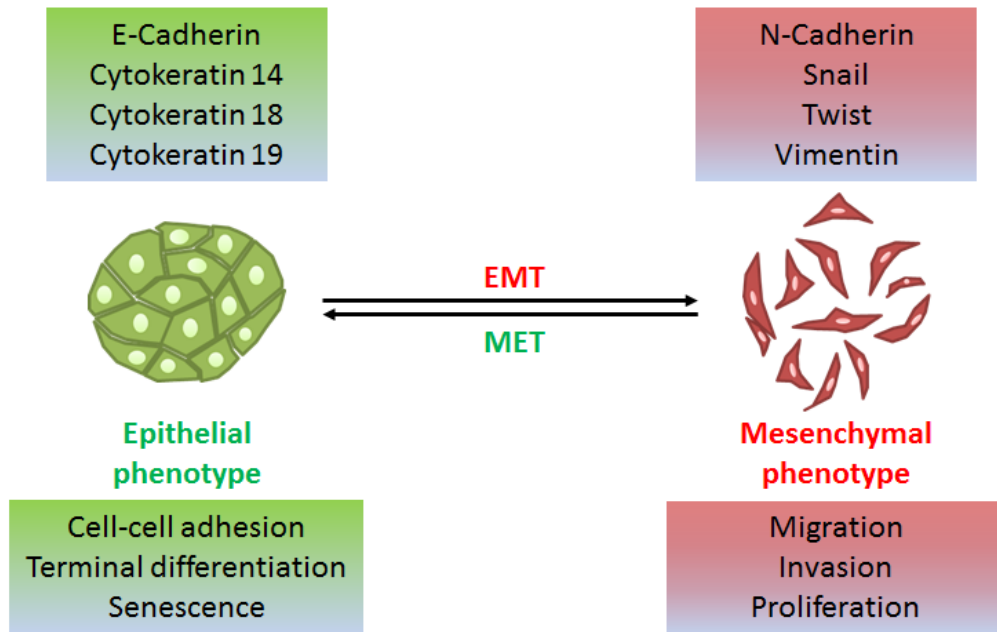


Figure 1.8: Epithelial-Mesenchymal Transition The epithelial phenotype is characterised by a rounded cell morphology, cell-cell adhesion, decreased cell proliferation and increased cell death whereas the features of Mesenchymal phenotype include increased proliferation, migration and invasion. The two phenotypes are also distinguished by their expression of specific epithelial and mesenchymal markers. Cells can transition from epithelial to mesenchymal phenotype or vice versa, depending on genetic or epigenetic changes or conditions in the microenvironment.

ther, pro-angiogenic factors are secreted by mesenchymal cancer cells, which enable the metastatic tumours to form new blood vessels, thus increasing their survival in the new environments they colonized[136].

1.4.1 Key proteins in EMT

E-box transcription factors

Three types of transcription factors are crucial in regulating EMT: the ZEB proteins ZEB1 and ZEB2, the Snail proteins Snail1, Snail2 and Snail3, and the basic helix-loop-helix (bHLH) proteins Twist1 and Twist2. All of them act by bind-

ing to a regulatory motif in the promoter regions of target proteins called the E-box which has a consensus sequence CANNTG. Binding of E-box factors to a promoter region results in either repression or activation of the target gene depending on their interaction with polycomb repressor complex (PRC) (among others) or p300/PCAF (p300/CBP associated factor) respectively. These transcription factors repress the expression of the epithelial proteins, prominently E-cadherin and cytokeratins while at the same time inducing the expression of various mesenchymal proteins including vimentin, vitronectin, fibronectin, collagen and matrix metalloproteinases[137].

E-cadherin

E-cadherin is a membrane protein of the cadherin superfamily and is a component of the adherens junction (zonula adherens). The extracellular domains of E-cadherin form one half of the adherens junction and interact with the cadherin domains of the neighbouring cell. The intracellular regions of the protein interact directly with β -catenin and indirectly, through α -catenin, with actin filaments. E-cadherin acts a crucial regulator of the Wnt signalling pathway by sequestering β -catenin; decreased expression of E-cadherin results in the release of β -catenin into the cytosol, which in turn results in its activation or degradation depending on the status of Wnt ligand signalling. E-cadherin is a tumour suppressor and its loss is causally implicated in breast, colorectal and ovarian cancers. Decreased expression of E-cadherin is a hallmark of EMT and is precursor of the destruction of the adherens junction and reorganization of the cytoskeleton[138].

Vimentin and cytokeratins

Vimentin and cytokeratins are components of intermediate filaments. Vimentin is expressed at high levels in mesenchymal cells and is responsible for maintaining the

structural integrity of the cell. It also helps in the transport of proteins across the intermediate filament. Cytokeratins, while performing a similar role in transport of proteins, differ from vimentin in one important respect: they transport E-cadherin to the cell membrane, enabling the establishment and maintenance of the epithelial phenotype[133].

1.4.2 Signalling pathways regulating EMT

TGF β signalling

TGF β signalling is a crucial regulator of EMT in cancer as well as development and wound healing. Activation of TGF β receptors by their ligands (TGF β 1 and TGF β 2) results in activation of Smad2/3, which in turn translocate to the nucleus and induce the transcription of a number of mesenchymal genes including Snail, Slug, ZEB1, ZEB2, collagen and matrix metalloproteinases. The activity of E-box-binding transcription factors can also be enhanced by interaction with Smad proteins[139].

Wnt signalling

When wnt signalling is not activated, β -catenin is bound to the destruction complex (consisting of axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3- β (GSK3 β) and casein kinase 1 α (CK1 α)) which targets it for ubiquitination and subsequent proteasomal degradation. Wnt ligands bind to the Frizzled receptor, inactivating the destruction complex, leading to the release of β -catenin and its consequent translocation to the nucleus. In the nucleus, β -catenin interacts with TCF4/LEF1 and helps in the transcription of target genes. E-cadherin negatively regulates Wnt signalling by directly binding to β -catenin, therefore preventing its translocation to the nucleus[140].

EGF signalling

Epidermal Growth Factor (EGF) binds to EGF receptor (EGFR) setting off a signalling cascade mediated by MAP kinase and PI3 kinase. EGF signalling also activates β -catenin, thus establishing a cross-talk with Wnt signalling. Ultimately, EGF signalling results in the transcription of E-box-binding proteins Snail, Slug, ZEB1 and ZEB2.

1.4.3 Role of EMT in bone metastasis

EMT is considered an essential process at various steps in the spread of cancer cells of epithelial origin to distant sites. As the cells of prostate cancer originate from the glandular epithelium, they exhibit strong cell-cell adhesions; the transformation of the cancer cells to a mesenchymal phenotype allows them to lose many of the features of epithelial cells, allowing them to become more invasive and promoting intravasation. These cells that enter the blood and lymphatic supply are called circulating tumour cells (CTCs). The role of EMT in the establishment of bone metastases is less clear. Cells of prostate cancer bone metastases have been shown to exhibit an epithelial phenotype similar to primary tumours, suggesting that once CTCs reach and colonize bone, the factors in the bone microenvironment induce mesenchymal-epithelial transition. This is corroborated in an *in vitro* experiment where ARCaPM cells co-cultured with HS-27a bone marrow stromal cells undergo mesenchymal-epithelial transition[141]. It is also thought that prostate cancer cells can exist as micrometastases in an epithelial state, and due to changes in the tumour microenvironment, undergo a mesenchymal-epithelial transition again, triggering a renewed cycle of invasion, proliferation and bone disease[142].

1.4.4 ARCaP cell line model of EMT

ARCaPE and ARCaPM cells are isogenic cell lines established from a single parental cell line ARCaP. ARCaP is a human cell line derived from peritoneal seeding in a prostate cancer patient. The androgen sensitivity status of these cell lines is unusual in that they are repressed by androgen signalling even though they express low levels of AR[143]. Ectopic overexpression of AR in ARCaP cells resulted in increased androgen-responsive tumour growth *in vitro* but decreased their metastatic potential *in vitro* and reduced tumour size in athymic nude mice[144]. From this, ARCaPE and ARCaPM were derived by clonal dilution *in vitro*[145][80](product datasheets, Novicure Inc, USA, Cat. 3423 and 3422). A further subclone, designated as ARCaPM1, was derived by passaging ARCaPM in SCID mouse bone metastasis model[79]. ARCaPE and ARCaPM cells differ significantly in their ability to form metastases in the intra-cardiac injection mouse model; ARCaPM cells have a high propensity to form bone and adrenal tumours following intra-cardiac injection whereas ARCaPE cells have a much lower propensity towards metastasis. ARCaPE cells exhibit an epithelial phenotype *in vitro*, characterized by a rounded morphology and high cell-cell adhesion (grow in clusters) and low migration. Conversely, ARCaPM cells are spindle-shaped, exhibit low cell-cell adhesion (spread out in culture) and possess a greater ability to migrate *in vitro*[145]. Treatment of ARCaPE cells with the growth factors TGF β and EGF results in EMT with a change to a mesenchymal morphology, decreased E-cadherin and increased vimentin and RANKL[80]. The effect of EGF was shown to be at least partially mediated by the hedgehog signalling molecule GLI1[146]. Similarly, exposure of ARCaPE cells to IGF1 results in upregulation of ZEB1 and EMT[147]. ARCaPE cells, when orthotopically inoculated in athymic nude mice, acquired increased heterogeneity and the derived subclones ex-

hibited a mesenchymal phenotype[148]. Further, treatment of ARCaPE cells with phorbol-12-myristate-13-acetate (PMA) induced EMT through the activation of protein kinase C (PKC)[149]. Overexpression of the zinc transporter protein LIV1 in ARCaPE cells results in EMT, and conversely, its repression in ARCaPM results in MET[150]. Inhibition of β 2-microglobulin also induces MET in ARCaPM cells[151]. It is thus clear from previous literature that ARCaPE and ARCaPM cells exhibit great plasticity in undergoing EMT and MET respectively following specific changes in their microenvironment and hence are a useful model to study EMT in prostate cancer.

1.5 MicroRNAs

MicroRNAs are 19-23 nucleotide-long small RNA molecules that regulate gene expression at the post-transcriptional level. Since their discovery in the late 1990's, microRNAs have been studied in great detail in human physiology and disease, particularly in cancers[152]. They have added an additional layer of complexity to regulation of gene expression and numerous reports have identified the crucial role microRNAs play in various cell signalling pathways in human development and disease. MicroRNAs have been found to be expressed in every tissue and cell type that has been examined so far.

1.5.1 Biogenesis

Up to 2000 microRNA genes have been identified so far. These genes may be discrete, encoding a single microRNA, or may be a cluster, with multiple microRNAs being expressed from a single locus. Further, genomic sequences encoding microRNAs may be present in the intronic regions of other coding or non-coding genes, termed as “host genes”. The majority of microRNA genes are transcribed by RNA polymerase II, generating a primary transcript called pri-mir or pri-microRNA.

The pri-mir may be up to several hundred nucleotides long. Within the nucleus, a ribonuclease complex consisting of the enzymes Drosha/Pasha cleave the pri-mir to a shorter 80-110 nucleotide sequence called precursor microRNA or pre-mir. This pre-mir has a characteristic stem-loop secondary structure, with partially complementary sequences at the ends folding back on themselves forming the “stem” and the intervening region constituting the “loop”. The pre-mir is actively transported out of the nucleus with the help of the Ran-GTPase complex. Once in the cytoplasm, the pre-mir is further cleaved by a sequence specific ribonuclease called Dicer, giving rise to an RNA double strand. One of these strands forms the predominant mature microRNA and is called the loading strand or miR, whereas the complementary strand, called the passenger strand or miR* is degraded. The strands formed from the 3’ and 5’ ends end of the pre-mir are designated as miR-3p and miR-5p respectively. The selection of -3p or -5p as the predominant mature microRNA depends on the specific microRNA, but also varies in different cell and tissue types[153].

1.5.2 Function

The mature microRNA is loaded on to the microprocessor complex, consisting of various ribonucleotide-binding proteins (RNPs). When the microRNA binds to its cognate sequence on a target messenger RNA, the RNPs of the microprocessor complex, Argonautes most importantly, help recruit ribonucleases to the miR-mRNA binding site. The target mRNA is then degraded; however, in the initial stages of microRNA binding, only translational inhibition may occur without mRNA degradation. Functional microRNA binding sites are generally present in the 3’ untranslated region (UTR) of the target mRNA, although binding sites in the 5’ UTR and coding region have also been identified. Many microRNAs

can target a single mRNA and conversely, one microRNA can target multiple mRNAs[154].

1.5.3 MicroRNAs in prostate cancer

A number of microRNAs have been found to be deregulated and have a functional role in prostate cancer progression. Mir-221/-222 are known to be expressed at higher levels in androgen-insensitive prostate cancer compared to androgen responsive tumours and cell lines. MiR-21 is another oncogene that is overexpressed in prostate cancer, in addition to a number of other cancers like those of the breast, lung and pancreas. Other oncogenic microRNAs include miR-125b, -32 and -148a. The expression of tumour suppressor microRNAs, in contrast, is reduced in primary and metastatic prostate cancer. The microRNAs miR-101, -126*, -330, -15, -16 and let-7 are all known to have a tumour suppressor function in prostate cancer[155][156][157]. More recently, microRNAs have been shown to be of use as blood-based biomarkers for diagnostic or prognostic classification of prostate cancers[158]. High serum levels of the microRNAs miR-141 and miR-375 were shown to be associated with metastatic prostate cancer[159].

1.5.4 MicroRNAs regulating EMT in prostate cancer

Non-coding RNAs like those of the miR-200 family can inhibit EMT and induce MET. MiRs-200a, -200b, -200c directly target mesenchymal transcription factors Snail, Slug, ZEB1 and ZEB2 and prevent their protein production post-transcriptionally. Members of the miR-200 family act as tumour suppressors and their expression is decreased in breast, prostate and other cancers[160][161][162]. MiR-203 and miR-205 are important mediators of EMT in prostate cancer. MicroRNA profiling in primary vs bone metastatic prostate cancer specimens identified miR-203 and miR-205 to be significantly downregulated[163][164][165].

1.6 Prostate cancer animal models

1.6.1 Tumourigenesis models

Mice never naturally acquire prostate cancer; it is thought that this may be due to their short life span, considering that the human disease commonly occurs in the later part of life. Hence, prostate cancer tumorigenesis is studied in transgenic mice tumour suppressor deficiency or oncogenic overexpression. The TRAMP mouse model was generated by overexpressing the SV40 large T antigen under the probasin promoter, whose expression is specific to the prostate gland. This model exhibits many of the features of human disease progression, including intra-epithelial neoplasia, cancer, hormone refractory tumours and metastasis. Similarly Myc transgenic mice also develop PIN and cancer. Heterozygous knockout of PTEN in mice results in tumours at multiple sites. However, in the prostate, changes are limited to epithelial hyperplasia and dysplasia indicating that heterozygous PTEN mutations alone are insufficient to produce malignant tumours. Other models like NKX3.1 knockout mice, androgen receptor and AKT transgenic mice have been used to study prostate cancer tumorigenesis and progression. However, none of these models lead to bone metastases, failing to recapitulate a very important feature of aggressive human prostate cancer[18].

1.6.2 Xenograft models

The xenograft models allow the study of the metastatic process in prostate cancer. Subcutaneous injection of prostate cancer cells in immune-deficient mice leads to tumour formation, but the tumours do not exhibit a distant spread. Intra-tibial models are used to specifically study prostate cancer-induced bone disease but are problematic due to the bone destruction at the site of injection. Prostate cancer cells (e.g. PC3, C4-2B, 22Rv1) are injected into the tibia of immune-

deficient mice, where they form tumours and importantly, form osteolytic or osteoblastic bone lesions. Intra-cardiac metastasis models, involve the injection of prostate cancer cells (PC3, ARCaPM, 22Rv1) into the left ventricle of immune-deficient mice[166][167]. Cells injected into the heart colonize distant sites (bone, liver, lungs, adrenals) which replicates the human disease to a greater extent. While the intra-cardiac models are limited to the study of the later stage of the metastatic process, precluding the study of early steps of cancer progression like local invasion and intravasation of primary tumour cells into the blood vessels and lymphatics, they do replicate the haematogenous spread of the tumour cells, extravasation and colonisation in distant organs and the associated tumour-stromal interactions. Both intra-tibial and intra-cardiac mouse models allow the study of prostate cancer-induced bone disease. Depending on the cell line used, the bone lesions formed at the tumour sites may be osteolytic, osteoblastic or mixed. PC3 cells have been most commonly used for *in vivo* studies due to their highly aggressive nature and relatively quick tumour formation, the bone lesions formed are exclusively osteolytic. As this is dissimilar to the mixed (osteoblastic + osteolytic) bone disease in human prostate cancer patients, various groups have experimented with other cell lines to more closely model the human disease. ARCaPM or 22Rv1 cells, when injected intracardially, produce bone tumours associated with mixed osteoblastic/osteolytic lesions[79][169]. A possible drawback in the use of immunodeficient mice is that the lack of host immunity may promote tumour progression and aggressiveness[170]. Despite this, xenograft mouse models have provided a wealth of information about the mechanisms involved in prostate cancer bone metastasis.

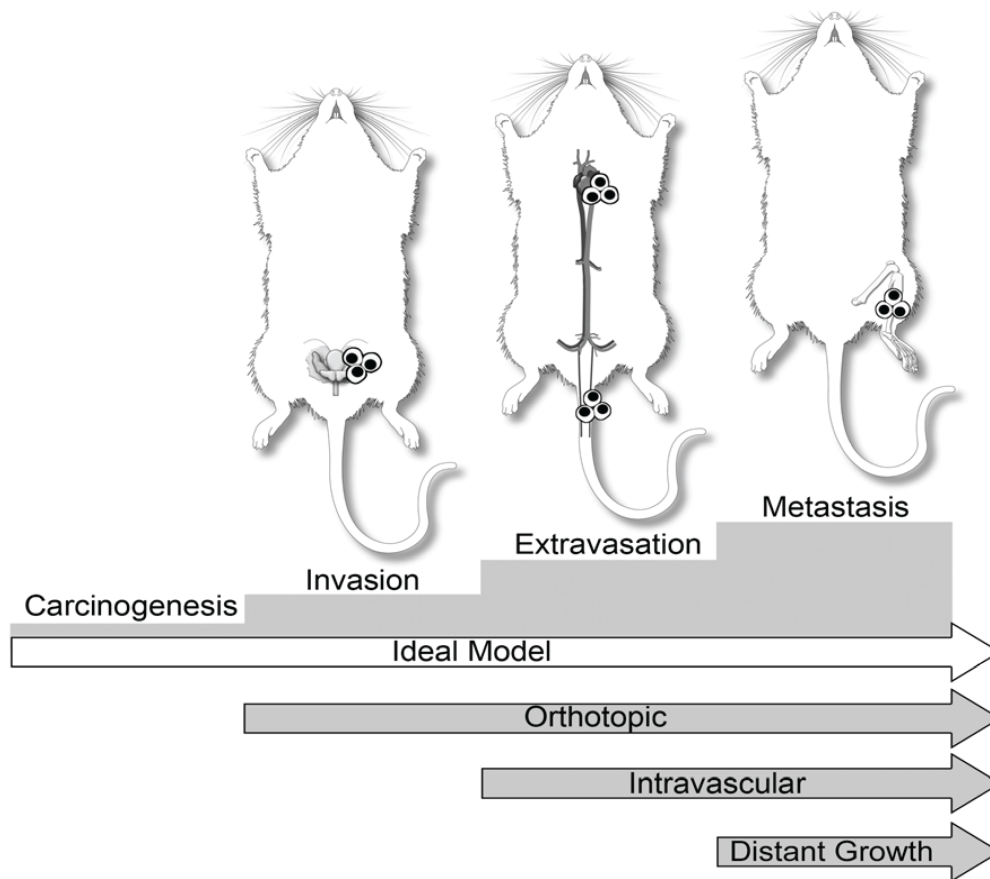


Figure 1.9: Xenograft mouse models of prostate cancer Different xenograft mouse models allow study of distinct stages of prostate cancer progression and metastasis. Orthotopic inoculation tries to replicate the disease from intravasation to distant metastasis, intra-cardiac or intravenous inoculation models allow study of extravasation and colonisation in distant sites and intra-tibial injection of tumour cells is a model of tumour formation and bone disease at the metastatic site. (Reproduced from Simmons et al.[168], Open access, CC BY-SA 3.0)

1.7 Osteomimicry

Prostate cancer cells are known to express markers specific to bone cells, including Runx2, bone sialoprotein, osteopontin, osteonectin and osteocalcin[171][172]. This is known as osteomimicry. Runx2 expression is elevated in bone metastatic human prostate cancer samples and is associated with increased aggressiveness in subclones of PC3 cells[173][83]. The expression of osteopontin is elevated in both primary and bone metastatic prostate cancers and correlates with MMP9 activity; knockdown of osteopontin in PC3 cells results in decreased colony formation and apoptosis[174][175][176][177][178]. The expression of osteocalcin protein is increased in bone metastatic prostate cancer cells compared to primary tumours[179]. These reports show that a clear link exists between osteomimicry and prostate cancer progression and bone metastasis.

1.8 Hypothesis and aims

Overall goal

The overall aim of this thesis was to identify novel mechanisms controlling the function and regulation of osteomimicry in prostate cancer.

Hypothesis

It was hypothesized that the bone metastatic ability of prostate cancer cells is partly due to osteomimicry, allowing the tumour cells to express bone-specific factors that increase their survival and metastatic potential. ALPL was the main focus of this thesis as it is the least studied factor with respect to its direct role in prostate cancer cells themselves (as opposed to its role as a bone turnover marker). Further, given the pervasive role of microRNAs as major regulators of cell

signalling pathways, it was hypothesized that microRNAs regulate osteomimicry in prostate cancer.

Specific objectives

1. To determine the significance of *in vitro* mineralization and the role of alkaline phosphatase in prostate cancer cell survival and EMT (Chapter [3](#))
2. To determine the effect of ATP on EMT in prostate cancer cells, and whether ATP mediates the effects of alkaline phosphatase (Chapter [4](#))
3. To identify novel microRNA regulators of *in vitro* mineralization, ALP activity and EMT (Chapter [5](#))

All models are wrong, but some are useful.

—George Box, *Empirical Model-Building and
Response Surfaces*

2

Materials and Methods

2.1 Cell lines and reagents

ARCaP, ARCaPE and ARCaPM human prostate cancer cells lines were purchased from Novicure Inc, USA (Cat. 3421, 3423 and 3422 respectively). All three cell lines were routinely maintained in MCaP medium (Novicure, Cat. 3300) with 5% fetal bovine serum (FBS) and penicillin-streptomycin, unless otherwise indicated. C4-2B human prostate cancer cells were a kind gift from Prof. George Thalmann, University of Bern, Switzerland, and maintained in RPMI 1640 medium with 10% FBS, vitamins, non-essential aminoacids, L-glutamine, sodium pyruvate and penicillin-streptomycin (complete RPMI medium), unless otherwise indicated. PC3 human prostate cancer cell line, obtained from American Type Culture Collection (ATCC) were routinely maintained in complete RPMI medium (Sigma) containing 10% FBS and penicillin-streptomycin. All other prostate cancer cell lines were a kind gift from Dr. Richard Bryant, University of Oxford. 2T3 mouse pre-

osteoblast cells were obtained from ATCC and maintained in RPMI 1640 medium with 10% FBS and antibiotics, unless otherwise indicated. Where possible, cell lines were validated by genotyping. Periodic testing ensured the absence of mycoplasma contamination in all cell lines. Apyrase was obtained from Sigma (Cat. A6535), 1 unit of which will liberate 1.0 micromole of inorganic phosphorus per minute at pH 6.5 at 30 °C. Porcine alkaline phosphatase was obtained from Sigma (Cat. P4439), 1 unit of which will hydrolyze 1.0 micromole of p-Nitrophenyl Phosphate per minute at pH 9.8 at 37 °C.

2.2 Transfection

2.2.1 Plasmid DNA transfection

Plasmid DNA was transfected using Lipofectamine 2000 (Life Technologies, Cat. 11668019) according to the manufacturer's protocol. Typically, 24 μ g of plasmid DNA in 1 ml of Opti-MEM (Life Technologies, Cat. 31985062) was mixed with 60 μ l of Lipofectamine 2000 in 1ml of Opti-MEM and incubated for 5-10 minutes at room temperature. The DNA-lipofectamine complexes were then added to cells at 80-90% confluence in 10 ml of antibiotic-free growth medium.

2.2.2 siRNA and microRNA mimic transfection

SiRNA or microRNA mimics were transfected in prostate cancer cells using Lipofectamine RNAiMax (Life Technologies, Cat. 13778-150) according to the manufacturer's protocol. Briefly, cells at 80-90% confluence in a 96-well plate were grown in antibiotic-free medium. Then, siRNA or microRNA mimics (5 pmol/well) in 5 μ l of Opti-MEM were mixed with Lipofectamine RNAiMax (0.3 μ l per well) in 5 μ l of Opti-MEM and incubated at room temperature for 5 minutes to form nucleic-acid complexes, which were then added to the target cells. De-

tails of siRNAs are as follows: non-targeting control pool: Cat. D-001206-13-05, ALPL-targeting ON-TARGETplus siRNA pool: Cat. L-008658-00-0005, SNAI1-targeting ON-TARGETplus siRNA pool: Cat. L-010847-01-0005, non-targeting control microRNA mimic: CN-001000-01-05, miR-373 mimic: Cat. C-300680-03-0002, all of which are from Dharmacon, GE Healthcare (formerly from Thermo Scientific).

2.3 Lentiviral packaging and transduction

Lentiviral packaging was performed using 293T cells using a 3rd generation packaging system. Briefly, 10 μg of transfer vector, 5 μg of pMDLg/pRRE, 2.5 μg of pRSV-Rev and 2.5 μg of pCMV-VSVG were transfected into 293T cells using Lipofectamine 2000 in a 10 cm tissue culture dish and 3 days later, the culture supernatant containing the lentivirus was collected. The lentivirus was made into a 10 times concentrated stock using the Lenti-X Concentrator Solution (Clontech, Cat. 631232) and was used at a dilution of 1:10 in the cell suspension for transduction. Hexadimethrine bromide (Sigma, Cat. H9268) was added at a concentration of 10 $\mu\text{g}/\text{ml}$ in the cell suspension to enhance transduction efficiency. The transfer vectors are scrambled control (Sigma, Cat. SHC002) or ALPL targeting short-hairpin RNAs (Sigma, Cat. SHCLNG-NM.000478; TRCN0000052003: CCGGCCCA-CAATGTGGACTACCTATCTCGAGATAGGTAGTCCACATTGTGGGTTTTTG, TRCN0000052004: CCGGCGTGGCTAAGAATGTCATCATCTCGAGATGATGACATTCTTAGCCACGTTTTTG, TRCN0000052005: CCGGGCGCAAGAGACACTGAAATATCTCGAGATATTTCAAGTGTCTCTTGCGCTTTTTTG, TRCN0000359258: CCGGATGTCTCCATGGTGGACTATGCTCGAGCATAGTCCACCATGGAGACATTTTTTG, TRCN0000359332: CCGGAGTATGAGAGTGACGAGAAAGCTCGAGCTTTCTCGTCACTCTCATACTTTTTTG), designated as shSCR and shALPL1-shALPL5 respectively. These plasmids have a pLKO.1

backbone, which is suitable for both 2nd as well as 3rd generation packaging systems.

2.4 RNA isolation

Total RNA was isolated from cultured cells using Trizol Reagent (Life Technologies, Cat. 15596-026) according to the manufacturer's protocol. Typically, adherent cells cultured in 24-well plates were used for RNA isolation. At the desired time point, the culture medium was discarded, 250 μ l of Trizol was added to each well to lyse the cells and homogenised by repeated pipetting. 50 μ l of chloroform was then added to the lysate and mixed thoroughly by shaking vigorously, followed immediately by centrifugation at 12000xg/4°C/15 minutes. The aqueous phase was carefully transferred to a fresh tube, 125 μ l of 2-propanol (isopropanol) was added, and mixed thoroughly by repeated inversion. The solution was allowed to incubate at room temperature for 10 minutes and then centrifuged at 12000xg/4°C/10 minutes. A pellet of RNA could be seen at this stage. The supernatant was discarded and the RNA pellet was washed with 70% ethanol in diethylpyrocarbonate(DEPC)-treated water. The tube was centrifuged at 7500xg/4°C/5 minutes, the supernatant was discarded, and the pellet was air-dried for 5-10 minutes. The RNA pellet was dissolved in 40-50 μ l nuclease-free DEPC-treated water. The isolated RNA contains both the small RNA fraction as well as the mRNA fraction and was quantitated using a Nanodrop. Quality of the RNA was assessed by measuring the ratios of absorbance (A260/A280 and A260/A230); ratios greater than 1.8 were considered suitable for further analysis.

2.5 Real-time quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

MicroRNA quantitative real-time PCR was performed as described in the Taqman microRNA Assays (Life Technologies) protocol. Briefly, 1 μ g of RNA was treated with DNase I (Fermentas, Cat. EN0521) to eliminate any contaminating genomic DNA and 10 ng of this RNA was used for reverse transcription using Taqman microRNA Reverse Transcription Kit (Life Technologies, Cat. 4366596). Real-time PCR was performed with these samples using Taqman microRNA primers (Life Technologies) using Taqman Universal Mastermix (Life Technologies, Cat. 4440041). For qRT-PCR of mRNA, 1 μ g of DNase I-treated RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Cat. 170-8891). This cDNA was appropriately diluted and was used in a qPCR reaction with Fast SYBR Green Real-time Mastermix (Life Technologies, Cat. 4385616) and Quantitect real-time PCR primers (Qiagen). Primer details are listed in Table [2.1](#).

Table 2.1: Primer sequences

Gene symbol	Forward primer	Reverse primer
ALPL	ACGTGGCTAAGAATGTCATC	CTGGTAGGCGATGTCCTTA
CDH1	AGGCCAAAGCAGCAGTACATT	ATTCACATCCAGCACATCCA
GAPDH	QuantiTect Primer Assay #QT01192646 (Qiagen)	
KRT14	GGCCTGCTGAGATCAAAGACT	TCTGCAGAAGGACATTGGCAT
P2RX7	AGTGCGAGTCCATTGTGGAG	CGCAGGTCTTGGGACTTCTT
SNAI1	QuantiTect Primer Assay #QT00010010 (Qiagen)	
RUNX2	QuantiTect Primer Assay #QT00020517 (Qiagen)	
VIM	CCTTGAACGCAAAGTGGAATC	GACATGCTGTTCCCTGAATCTGAG
ZEB1	CCAGGGAGGAGCAGTGAAAG	CCCCAGGATTTCTTGCCCTT
RNU6B	Cat # 440887 ID: 001093 (Life Technologies)	
hsa-miR-203	Cat # 440887 ID: 000507 (Life Technologies)	
hsa-miR-373	Cat # 4427975 ID: 000561 (Life Technologies)	

2.6 Protein isolation and western blotting

Cultured cells were washed gently with 1X PBS at room temperature to remove residual growth medium and the recommended amount (for example, 300 μ l for each well of a 6-well plate) of CellLytic M cell lysis reagent (Sigma, Cat. C2978) plus protease inhibitor cocktail (Sigma) was added to the wells. After incubation at room temperature for 30 minutes with periodic shaking, the cell lysates were centrifuged at 12,000xg for 15 minutes to pellet the cell debris. The supernatant was transferred to a fresh container and the lysates were stored at -20°C until further use.

The concentration of protein was quantified using a Pierce bicinchonic acid (BCA) assay kit (Life Technologies, Cat. 23225). This involves a colorimetric reaction which was read on a plate-reader at 562 nm wavelength and protein concentrations were calculated using standard curves obtained from serial dilutions of a bovine serum albumin (BSA) solution (provided in the kit).

These total protein samples were denatured in a denaturing buffer and 20 μ g each of the samples was resolved in 4-20% gradient sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred on to PVDF membranes (Millipore) using a semi-dry transfer system (Transblot, Bio-Rad). The membranes were blocked for 1 hour against non-specific binding with 5% semi-skimmed milk in tris-buffered saline with 0.2% Tween (TBST), and incubated 1:1000 dilution of E-cadherin or 1:5000 dilution of β -actin primary antibodies (Cell Signalling, Cat. 3195 and Sigma, Cat. A5316 respectively) in TBST at 4°C overnight, while rocking. Following this, the membrane was washed in TBST 3 times for 10 minutes duration each, followed by incubation for 1 hour at room temperature with the secondary antibody (anti-rabbit or anti-mouse respectively) (Cell Signalling). The membrane was washed 3 times again as above and the Novex ECL Chemilumines-

cent Substrate Reagent Kit (Life Technologies, Cat. WP20005) was used to detect the signal on an x-ray film. The intensity of protein bands was measured using ImageJ[180].

2.7 Cell viability

Viability of cultured cells *in vitro* was determined using the Alamar Blue assay. Briefly, a working solution (1 mg/ml) of resazurin (Sigma, Cat. R7017) was added to the cultured cells at a ratio of 1:10 with respect to the volume of growth medium (10 μ l in each well of a 96-well plate containing 100 μ l of growth medium). The cells were then incubated in the humidified CO₂ incubator at 37 °C until a change in colour was visibly noticeable and the plate was read using a fluorimeter (Fluostar) (excitation: 545 nm, emission: 585 nm). From the measured raw fluorescence values, the values from blank wells (growth medium with Alamar Blue but without cells) were subtracted to eliminate background fluorescence.

2.8 YO-PRO-1 staining

YO-PRO-1 iodide stain (Life Technologies, Cat. Y3603) (stock concentration: 1 mM in DMSO) was added to the growth medium of cells growing in 96-well plates, to a final concentration of 1 μ M (1:1000 dilution). The cells were then incubated in the humidified CO₂ incubator at 37 °C for 60 minutes and the plate was read using a fluorimeter (excitation: 490 nm, emission: 510 nm). From the raw readings, the values from blank wells (growth medium with YO-PRO-1 without cells) were subtracted to eliminate background fluorescence.

2.9 Alkaline Phosphatase (ALP) activity assays

2.9.1 Staining for alkaline phosphatase activity

Staining for alkaline phosphatase activity was performed using the Leukocyte Alkaline Phosphatase Kit (Sigma, Cat. 85L1) according to the manufacturer's protocol. Briefly, a solution of Fast Blue RR salt was made by dissolving the contents of 1 capsule in 48 ml of deionised water. A working solution was prepared by mixing Naphthol AS-MX solution to Fast Blue RR solution (2:48 ml ratio). Cells were fixed in tissue culture plate with 10% Formalin for 10 minutes at room temperature, washed once with deionised water and the working solution of the stain was added to the plate. It was then incubated at room temperature for 30 minutes in the dark. The wells were subsequently washed once with deionised water and the plate was scanned with a flat-bed scanner; blue staining indicates ALP activity.

2.9.2 Quantitative measurement of alkaline phosphatase activity

Cell lysates were obtained as described above. A 20X stock solution was made by dissolving 1.025mg/mL of 4-methylumbelliferyl phosphate (substrate for alkaline phosphatase; Sigma, Cat. M8168) in 50mM Tris (pH 8) buffer. A working solution was made by diluting the stock solution with 50mM Tris (pH 8) buffer. To 60 μ l cell lysate or conditioned medium in an opaque 96-well plate, 100 μ l of working substrate solution was added and incubated in the dark at 37 °C for 45 minutes. Subsequently, the reaction was stopped by adding 100 μ l of 1M Na₂CO₃ and the plate was read in a fluorimeter at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The fluorescence reading is proportional to alkaline phosphatase activity.

2.10 Mineralization assay

For mineralization assays, cells were grown in osteogenic medium (20 mM β -glycerophosphate, 50 mM ascorbic acid in α -MEM with 10% FBS) for 2-3 weeks or as indicated; osteogenic medium was changed every 3 days. At the end of the experiment, cell culture medium was discarded and the adherent cells at the bottom of the wells were fixed with 10% formalin for 10-15 minutes. Alizarin Red S (ARS) staining: After fixation with formalin, the wells were washed once with deionised water and Alizarin Red S solution (40 mM in water, pH 4.1-4.3) was added to the wells to completely cover the cell layer. After incubation in ARS solution for 30 minutes, the wells were washed with deionised water 3 times, photographed under a Nikon inverted microscope and the entire plate was scanned on a flat-bed scanner.

2.11 Small RNA-sequencing

RNA from cells was extracted by the TRIzol method as described above and library preparation, small RNA-sequencing and data analysis was performed on the Illumina platform at the Wellcome Trust Centre for Human Genetics, University of Oxford.

2.12 Statistics

All statistical analyses were performed using Graphpad Prism. Comparisons between 2 groups of data were performed using two-tailed unpaired Student's t test. Variances of data between the two groups were compared using an F test; where there was unequal variance between the two groups, Welch's correction was used. For comparisons between more than 2 groups, multiple t test with Holm-Sidak correction was used. In all cases, significance was assumed at $\alpha < 0.05$. For small

RNA-sequencing data, p values were adjusted using Bonferroni's correction over the raw p values.

*Life is full of mystery, yeah
But there are answers out there
And they won't be found
By people sitting around
Looking serious
And saying, isn't life mysterious?*

—Tim Minchin, *Storm*

3

Alkaline phosphatase regulates EMT and cell survival in bone metastatic prostate cancer cells

3.1 Introduction

3.1.1 Osteomimicry

Prostate cancer cells are known to express osteoblast markers and this is termed as osteomimicry (described earlier in Chapter 1 [1.7](#)). Koeneman et al.[\[171\]](#) proposed that osteomimicry may help prostate cancer cells to survive better in the bone microenvironment. A number of osteoblast-specific genes are expressed in prostate cancer cells and many reports have demonstrated their functional role in prostate cancer progression and metastasis(summarised in Table [3.1](#)).

Table 3.1: Role of osteoblast-specific genes in prostate cancer

Osteoblast marker	Physiological function	Role in PCa	Reference
Alkaline phosphatase	Mineral deposition	(unknown)	
Runx2	Transcription factor	Pro-metastatic	[83][181]
Osteopontin	organic matrix	proliferative, pro-invasive, pro-metastatic	[174][175][176][177][178]
Bone Sialoprotein	organic matrix	pro-metastatic	[182][183][184]
Osteocalcin	organic matrix	Pro-metastatic	[179]
Osteonectin	organic matrix	inhibits growth, pro-metastatic	[185][186][187]
Collagen	organic matrix	pro-migratory, pro-invasive	[188][189]

3.1.2 *In vitro* mineralization in prostate cancer cells

Mineral deposition *in vitro* is widely considered as evidence for osteoblast function. It has been used as a defining functional assay for the confirmation of primary osteoblasts as well as in differentiation studies using pre-osteoblasts or mesenchymal stem cells. Addition of osteogenic factors (e.g. BMP2, ascorbic acid) to the growth medium of cultured cells results in the differentiation of pre-osteoblasts to mature osteoblasts; β -glycerophosphate is a source of organic phosphate from which alkaline phosphatase catalyses the formation of calcium phosphate (the calcium is derived from the growth medium). Osteoblasts obtained from ALPL knockout mice are unable to deposit calcium phosphate *in vitro*, demonstrating the importance of the enzyme in *in vitro* mineralization[53]. Due to their osteotropic nature, *in vitro* mineralization of prostate cancer cells has been a focus of a number of studies. C4-2B cells, which are a derivative of LNCaP, are able to form osteoblastic bone lesions in intra-tibial mouse models. Lin et al[190] showed that they can mineral-

ize *in vitro* and also express alkaline phosphatase in addition to other osteoblast markers. 22Rv1 cells, a derivative of CWR22 cells, can also form bone 'spicules' at the site of tumours in intra-tibial mouse models. Further, the majority of prostate cancer cells can mineralize *in vitro* whereas non-cancer prostate cell lines do not exhibit this behaviour[191].

3.1.3 Alkaline phosphatase (bone/liver/kidney) (ALPL)

ALPL or tissue non-specific alkaline phosphatase (TNAP) is an enzyme that is, as the name implies, normally expressed in many tissues like bone, liver, kidney, lungs and leukocytes. ALP activity is considered an important marker of osteoblast activity as it catalyses the formation of the inorganic mineral part of the bone matrix. ALP activity in serum is also a marker of bone turnover in prostate cancer patients with bone metastasis. However, the expression and function of ALPL in prostate cancer cells themselves has never been explored.

3.2 Aim

In contrast to other major osteoblast markers, very little is known about the expression and role of ALPL in prostate cancer. Therefore, it was aimed to determine the effect of ALPL in prostate cancer cell biology, specifically on EMT and cell survival.

3.3 Methods

3.3.1 *In vitro* migration assay

Migration was measured *in vitro* using a "wound healing" assay. For this, cells were grown in 24-well plates in the desired conditions until they are fully confluent, and a scratch "wound" was made in the cell layer using a 20-200 μ l pipette tip.

Following this, floating cells were removed by gently washing the wells 3 times with 1X PBS, and fresh growth medium was added to the wells. The cells were incubated at 37 °C in a humidifying incubator with 5% CO₂ and microscopy images were obtained at 4X magnification every 6 hours up to a maximum of 48 hours.

3.3.2 Data mining

Gene expression data from the Cancer Cell Line Encyclopaedia (CCLE) and the Memorial Sloan-Kettering Cancer Center (MSKCC) datasets, available at cBioPortal[192] were mined using R statistical software[193]. Example code that was used to fetch the data from the MSKCC dataset is listed in Appendix B. To analyse correlation between various gene expression sets, Pearson's (r) and Spearman's (ρ) correlation or coefficients were used. A coefficient of $<+(-)0.36$ was considered as weak positive (negative) correlation, $+(-)0.36 - +(-)0.6$ as moderate positive (negative) correlation and $>+(-)0.6$ as strong positive (negative) correlation[194].

3.4 Results

3.4.1 ARCaPE/M cells are a model of epithelial-mesenchymal transition

ARCaPM cells form bone metastases *in vivo*, and in combination with ARCaPE, represent a good model to study EMT. To confirm that these cells were exhibiting the reported characteristics, we compared their morphology, cell death and markers of EMT. ARCaPE and ARCaPM cells differed in their shape in *in vitro* monolayer cultures: ARCaPE cells exhibited an epithelial morphology, characterised by a predominantly rounded appearance and increased cell-cell adherence evidenced by a tendency to grow in clusters (Fig. 3.1A). ARCaPM cells exhibited 50% less cell death, measured by YO-PRO-1 staining ($P < 0.05$) (Fig. 3.1B), compared to ARCaPE cells. ARCaPE cells have previously been reported to express higher

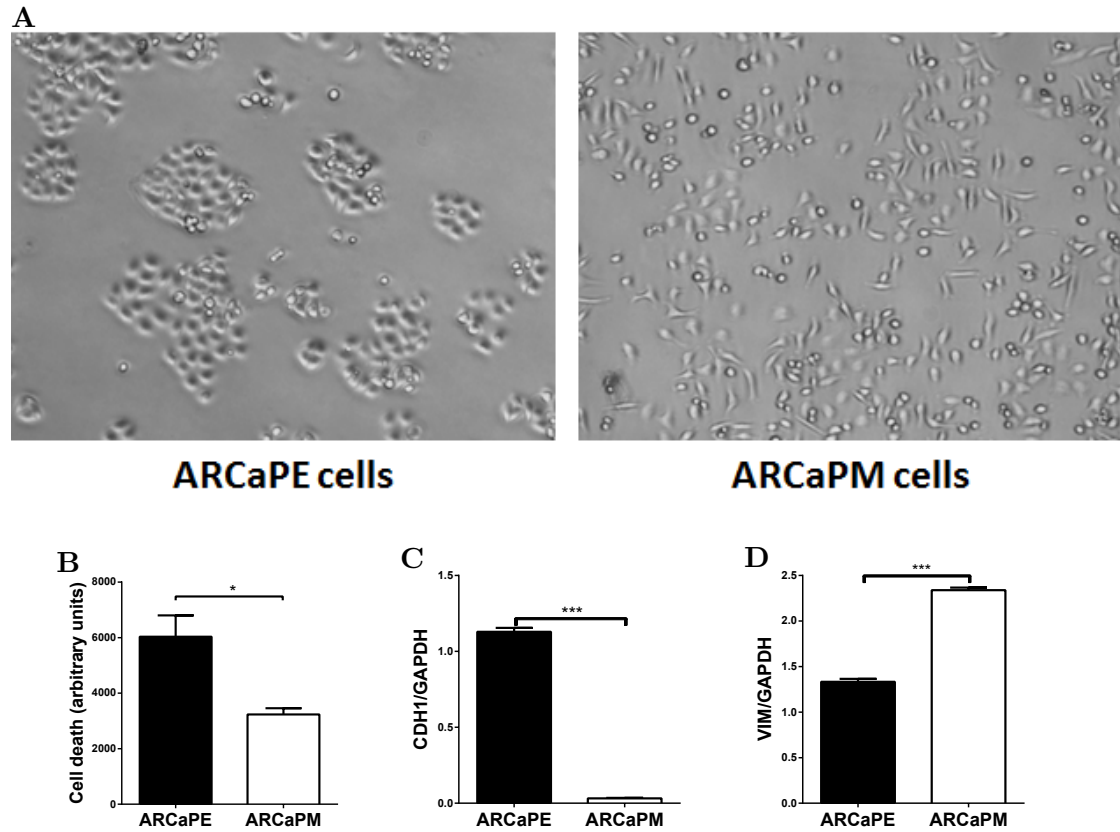


Figure 3.1: ARCaPE and ARCaPM cells are a model of epithelial-mesenchymal transition. (A) Morphology of ARCaPE and ARCaPM cells, under phase contrast microscopy at 200X magnification. (B) Cell death in ARCaPE and ARCaPM cells was quantified using YO-PRO-1 dye fluorimetry. (C) E-cadherin (CDH1) and (D) Vimentin (VIM) gene expression in ARCaPE and ARCaPM cells were measured by SYBR Green real-time PCR and normalized with GAPDH expression. ARCaPE and ARCaPM cells were grown for 72 hours in complete growth medium. (Data are representative of 3 independent experiments and shown as mean $\pm$ S. E. * $P < 0.05$, *** $P < 0.001$)

levels of epithelial markers and lower levels of mesenchymal markers compared to ARCaPM cells[80]. Expression level of E-cadherin mRNA was 95% lower ($P < 0.001$) (Fig. 3.1C) and vimentin mRNA 80% higher ($P < 0.001$) (Fig. 3.1D) in ARCaPM compared to ARCaPE.

3.4.2 ARCaPM cells express high levels of ALPL

The expression of osteoblast markers by prostate cancer cells is termed as osteomimicry. Alkaline phosphatase (ALP) is expressed in high amounts in osteoblasts and is considered a defining feature of mature osteoblasts. ARCaPM cells expressed a high level of ALPL mRNA whereas there was little or no expression in ARCaPE (P<0.001) (Fig. 3.2A). ALP activity was also significantly higher in ARCaPM cells compared to ARCaPE cells, measured both by a quantitative fluorescent assay (P<0.0001) (Fig. 3.2B) as well as qualitatively using a Leukocyte ALP staining kit (Fig. 3.2C). The mouse pre-osteoblast cells 2T3 are known to stain for ALP activity and were used as a positive control here[195].

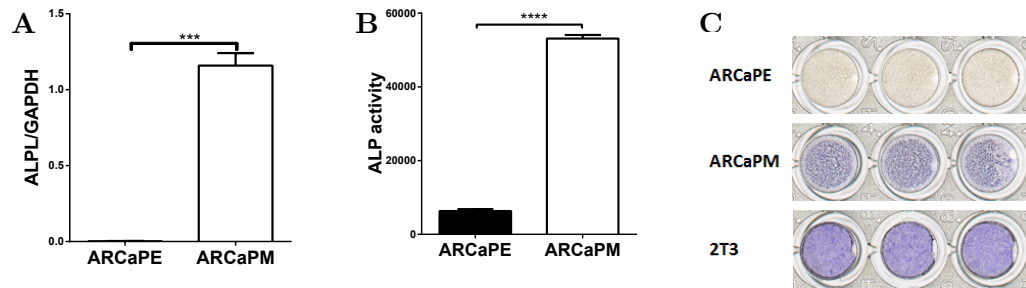


Figure 3.2: ARCaPM cells express high levels of ALPL mRNA and ALP activity compared to ARCaPE cells. (A) ALPL gene expression was measured by SYBR Green real-time PCR and normalized with GAPDH expression. (B) ALP activity in ARCaPE and ARCaPM cell lysates was measured using a fluorogenic ALP substrate and normalized with total protein concentrations. (C) ALP activity in confluent monolayers of ARCaPE, ARCaPM and 2T3 cells was measured using Leukocyte ALP staining kit. (Data are representative of 3 independent experiments and are shown as mean $\pm$ S. E. *** P < 0.001, **** P < 0.0001)

3.4.3 Differential expression of ALPL in a panel of prostate cell lines

ALPL mRNA expression was measured in a panel consisting of non-tumourigenic prostate (PNT1a, PNT2), non-metastatic prostate cancer (ARCaP, ARCaPE, LNCaP, MDA-PCa-2a, MDA-PCa-2b), bone-metastatic prostate cancer (ARCaPM, C4-2B, VCaP, PC3), prostate cancer cells that metastasize to sites other than bone (LNCaP-LN3, C4-2, DuCaP, Du145), breast cancer (MCF7) and bone marrow stromal (HS-5) cell lines (Table 3.2)(Fig. 3.3A). ARCaPM cells expressed the highest amount of ALPL among all prostate cell lines, followed by VCaP, DuCaP and PC3. The human bone marrow stromal cell line HS-5 also expressed a high level of ALPL. ALPL mRNA expression data in prostate cancer cell lines was also mined from the Cancer Cell Line Encyclopaedia (CCLE) dataset[196]. The CCLE dataset consists of 7 prostate cancer cell lines: 22Rv1, Du145, LNCaP, MDA-PCa-2b, NCIH660, PC3 and VCaP. In this dataset, ALPL expression was highest in 22Rv1 cells, followed by PC3, Du145 and VCaP (Fig. 3.3B).

Table 3.2: List of prostate cancer cells and their metastatic potential in animal models [197][198]

Cell line	Metastatic potential	Nature of bone lesions	Reference
ARCaP	moderate	NA	[143]
ARCaPE	very low	NA	[79]
ARCaPM	high	osteoblastic + osteolytic	[79]
LNCaP	very low	NA	[168]
LNCaP-LN3	moderate	NA	[168]
C4-2	high	NA	[168]
C4-2B	high	osteoblastic	[168]
VCaP	(unknown)	NA	[199]
DuCaP	(unknown)	NA	-
PC3	very high	osteolytic	[168]
Du145	low	NA	[200]
MDA-PCa-2a	(unknown)	NA	
MDA-PCa-2b	(unknown)	osteoblastic	[201]
PNT1a	NA	NA	-
PNT2	NA	NA	-
22Rv1	moderate	osteolytic + osteoblastic	[167][169]
NCIH660	(unknown)	NA	-

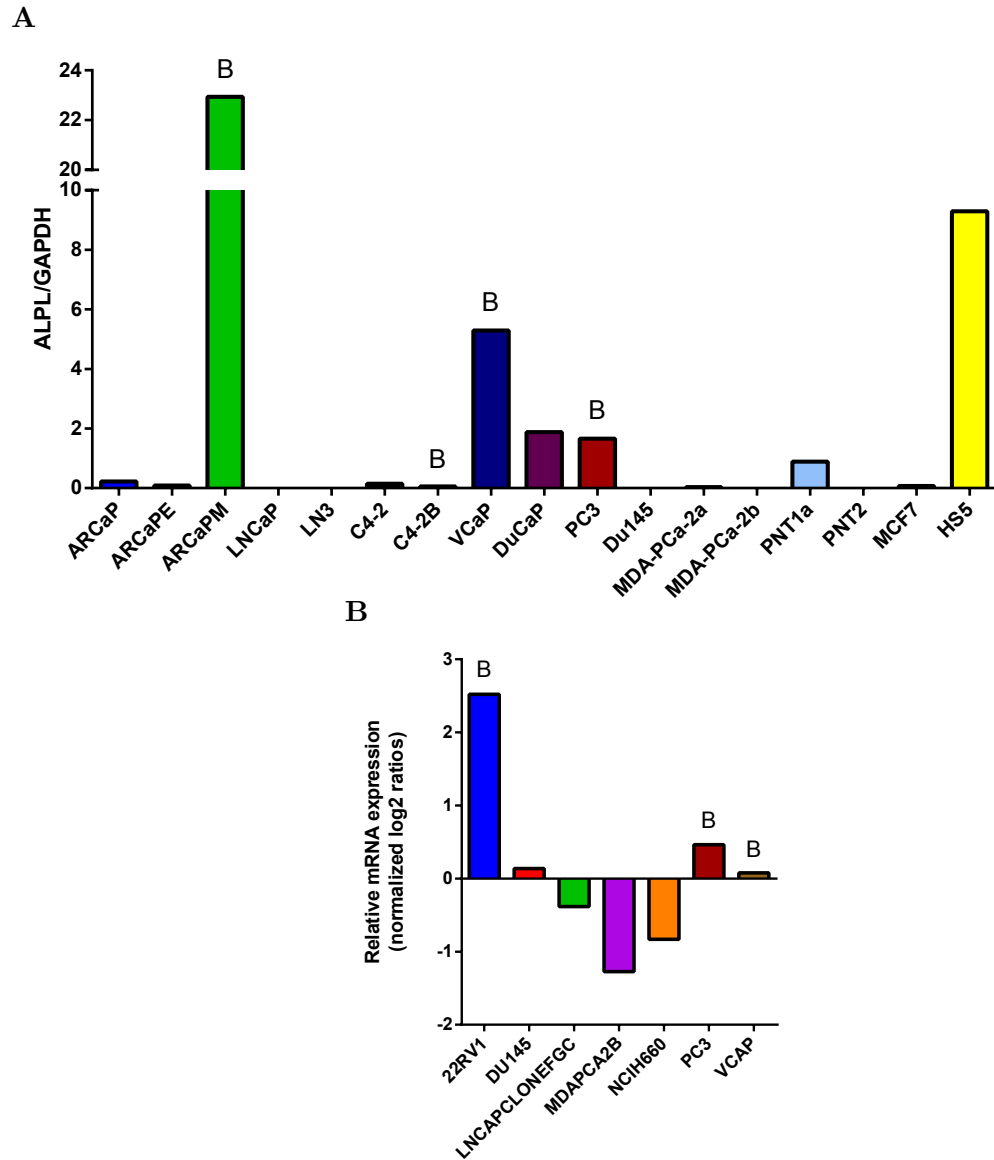


Figure 3.3: ALPL gene expression in prostate cancer cell lines (A) ALPL mRNA expression in a panel, consisting of non-tumourigenic prostate, non-metastatic prostate cancer, bone-metastatic prostate cancer, prostate cancer cells that metastasize to sites other than bone, breast cancer and myeloma cell lines, was measured using SYBR Green real-time RT-PCR and normalized with GAPDH expression. Data are shown as mean of 3 replicates. (B) ALPL mRNA expression data for a panel of 7 prostate cancer cell lines was mined from the Broad/Novartis Cancer Cell Line Encyclopaedia dataset on cBioPortal. “B” denotes cell lines that form bone metastases in *in vivo* models.

3.4.4 ARCaPM cells, but not ARCaPE, mineralize *in vitro*

In vitro mineralization is a defining feature of osteoblasts and alkaline phosphatase expressed by osteoblasts is crucial for this process. Therefore, we aimed to determine whether ARCaPE and ARCaPM cells differed in their ability to mineralize *in vitro*. ARCaPM cells were grown for 21 days in osteogenic medium (containing ascorbic acid and β -glycerophosphate), with the medium changed every 2-3 days, were stained intensely with Alizarin Red, indicating the presence of calcium deposits (Fig. 3.4A and 3.4B). In contrast, ARCaPE cells cultured under the same conditions did not mineralize. *In vitro* mineralization in 2T3 mouse pre-osteoblasts, although a faster process (10 days), produced a similar staining pattern as seen through the microscope (Fig. 3.4C).

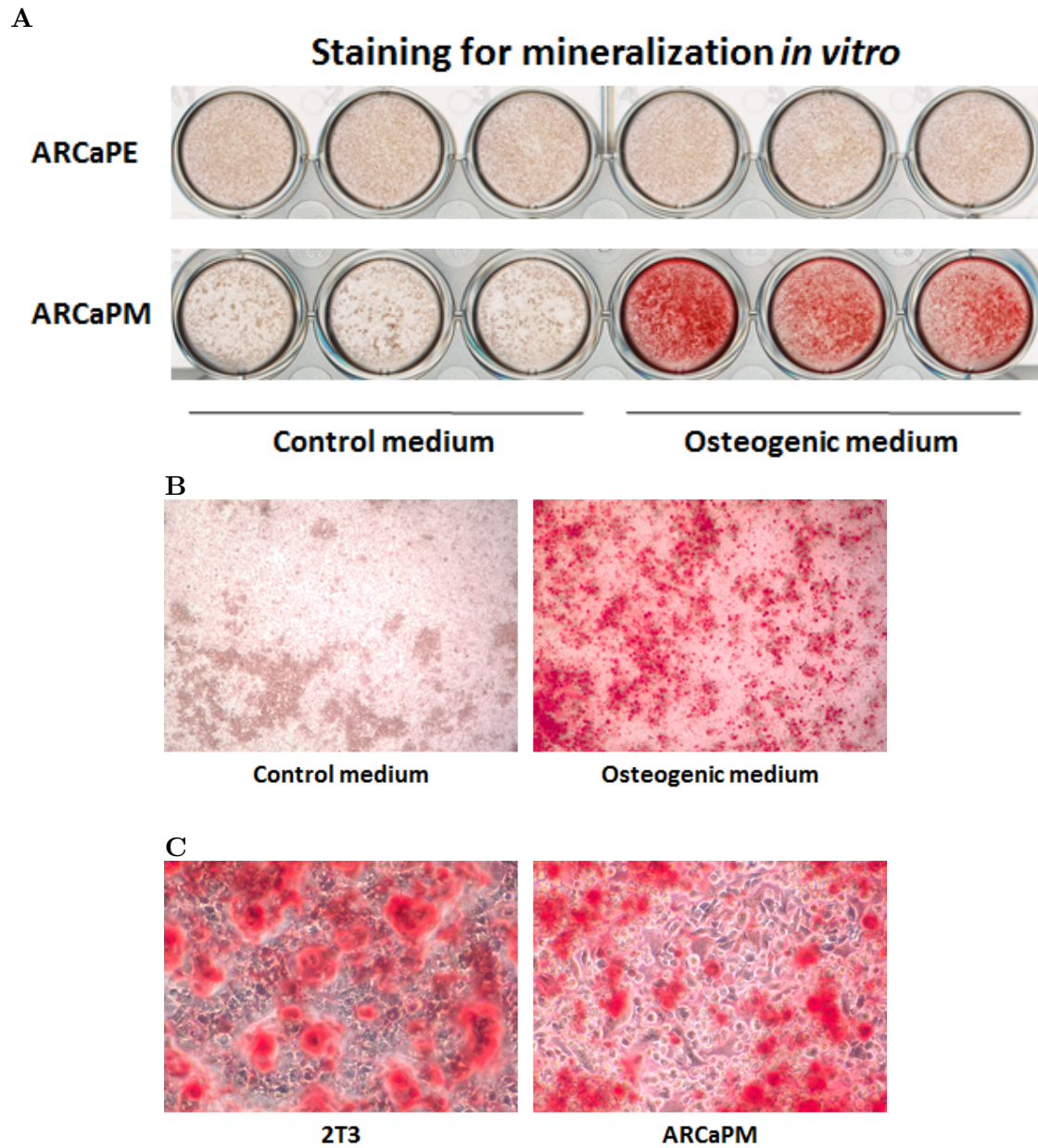


Figure 3.4: ARCaPM cells deposit inorganic mineral *in vitro* whereas ARCaPE cells do not mineralize. (A) ARCaPE and ARCaPM cells were grown in control or osteogenic medium for 21 days and stained (red) for mineral deposition using Alizarin Red S. (B) Bright-field microscopy (4X magnification) of ARCaPM cells in control or osteogenic medium, showing areas of mineral deposition in red. (C) Bright-field microscopy (20X magnification) of 2T3 mouse pre-osteoblasts and ARCaPM cells showing areas of mineralization in red. Images are representative of >5 independent experiments in triplicates.

3.4.5 Knockdown of ALPL induces change in morphology and induces cell death in ARCaPM cells

While *in vitro* mineralization is known to occur in C42B[190] and other prostate cancer cells[191], the role of alkaline phosphatase has never been explored in prostate cancer progression and metastasis. ALPL expression was knocked down in ARCaPM cells using lentiviral delivery of shRNA. Knockdown was confirmed by a significant decrease of ALPL mRNA levels by up to 90% (Fig. 3.5A) ($P < 0.05$) and a significant decrease in ALP activity by up to 80% (Fig. 3.5B) ($P < 0.001$). ALPL knockdown resulted in a marked change in the morphology of ARCaPM cells from a spindle-shaped, scattered appearance to a rounded shape and cells aggregating in clusters (Fig. 3.5C). Knockdown of ALPL in ARCaPM cells also decreased viability by 50%, measured by Alamar Blue assay (Fig. 3.6A) and increased cell death by 40-50%, measured by YO-PRO-1 staining (Fig. 3.6B).

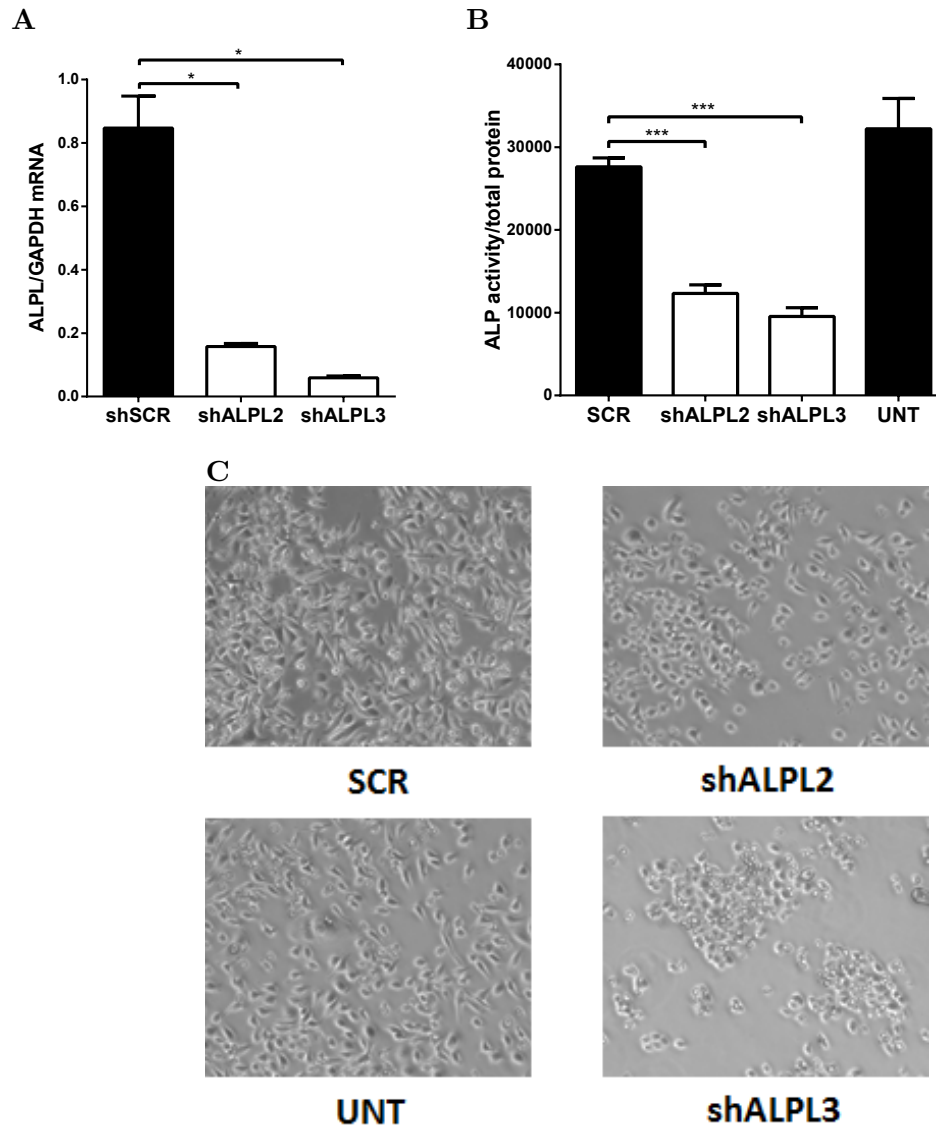


Figure 3.5: Efficient knockdown of ALPL in ARCaPM cells alters their morphology. (A) ALPL mRNA expression was measured in ARCaPM cells, 72 hours following lentiviral transduction of control (shSCR) or ALPL-targeting (shALPL2, shALPL3) shRNAs, using real-time RT-PCR and normalized with GAPDH expression. (B) ALP activity was measured in ARCaPM cells transduced with control or ALPL-targeting shRNA, using a fluorogenic substrate. (C) Morphology of control or ALPL knockdown ARCaPM cells imaged using phase contrast microscopy at 10X magnification. (Data are representative of 3 independent experiments and are shown as mean $\pm$ S. E. * $P < 0.05$, *** $P < 0.001$)

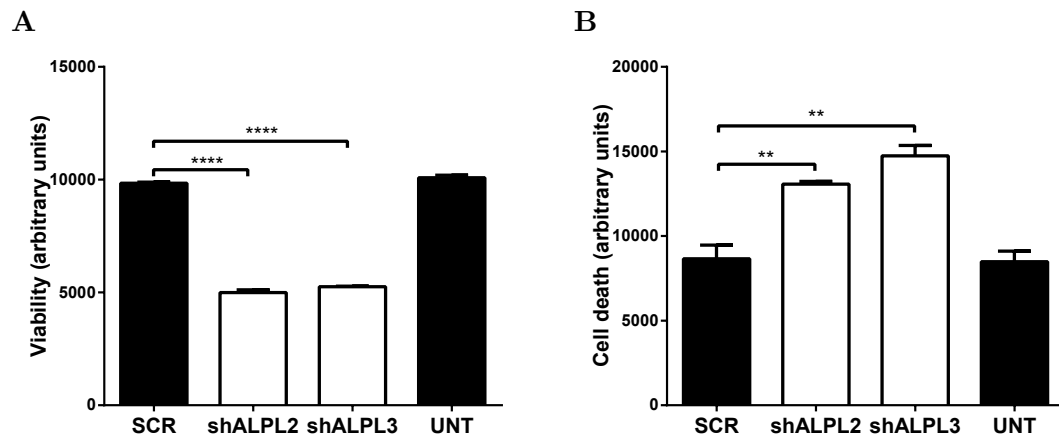


Figure 3.6: Efficient knockdown of ALPL in ARCaPM cells reduces cell viability and increases cell death. (A) Cell viability was measured in ARCaPM cells following ALPL knockdown (72 hours after lentiviral transduction) using Alamar Blue assay (B) Cell death was measured using YO-PRO-1 staining. (Data are representative of 3 independent experiments and are shown as mean $\pm$ S. E. ** $P < 0.01$, **** $P < 0.0001$)

3.4.6 Knockdown of ALPL reduces migration in ARCaPM

Knockdown of ALPL in ARCaPM cells resulted in significantly ($P < 0.0001$) decreased migration by up to 80% as seen in an *in vitro* scratch assay, quantitated using ImageJ software (Fig. 3.7).

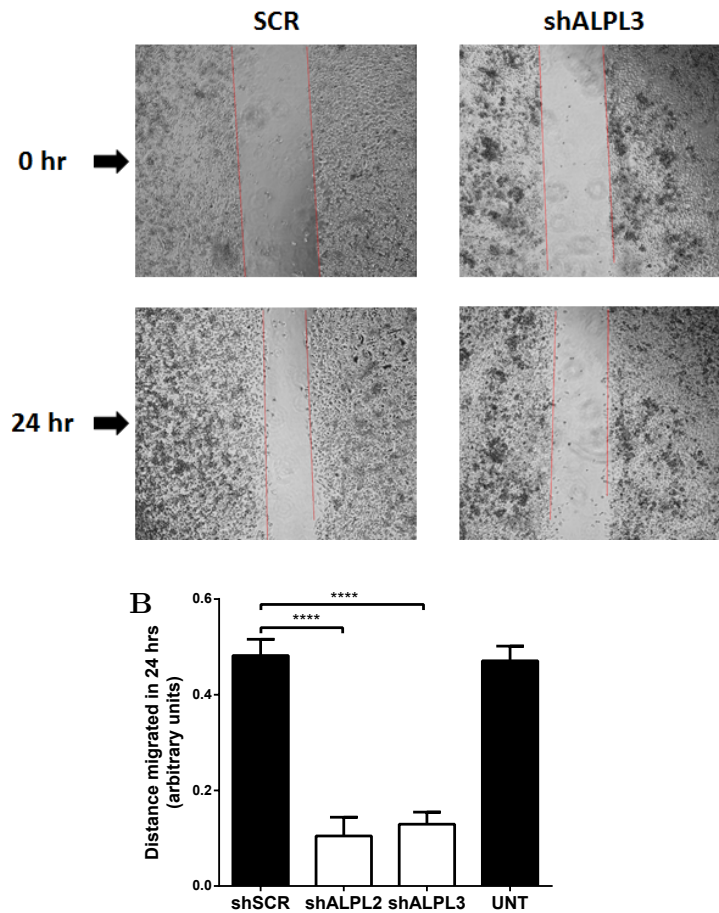


Figure 3.7: Knockdown of ALPL in ARCaPM cells decreases migration
 (A) Scratch assay following ALPL knockdown (48 hours after lentiviral transduction) in ARCaPM cells was imaged using phase contrast microscopy at 4X magnification. (B) Cell migration was quantified from the scratch assay using ImageJ analysis software to calculate distance migrated in 24 hours. (Data are representative of 2 independent experiments in triplicates, shown as mean $\pm$ S. E. **** $P < 0.0001$)

3.4.7 ALPL knockdown results in increased expression of E-cadherin

Transient transfection of siRNA targeting ALPL in ARCaPM resulted in a significant reduction in ALPL mRNA levels by 70% ($P < 0.0001$) (Fig. 3.8A). ALPL knockdown resulted in a significant ($P < 0.001$) increase in the expression of E-Cadherin mRNA by 1000% (Fig. 3.8B) and also an increase in E-Cadherin protein (Fig. 3.8C). However, there was no significant change in the expression of Vimentin or ZEB1 (Fig. 3.8D and 3.8E).

3.4.8 Knockdown of ALPL induces change in morphology, reduction in viability and increased cell death in PC3 cells

PC3 cells are often used as a model of bone metastatic prostate cancer in *in vivo* mouse models. In addition, this study revealed ALPL expression in PC3 cells. Hence, ALPL was knocked down using siRNA in PC3 cells; knockdown was confirmed by a reduction in ALPL mRNA expression, measured by SYBR Green real-time RT-PCR (Fig. 3.9A) ($P < 0.05$). This resulted in decreased Alamar Blue activity (Fig. 3.9B) ($p < 0.0001$) and increased YO-PRO-1 staining (Fig. 3.9C) ($P < 0.05$), indicative of decreased viability and increased cell death respectively. ALPL knockdown also altered PC3 cells from mesenchymal to epithelial morphology (Fig. 3.9D).

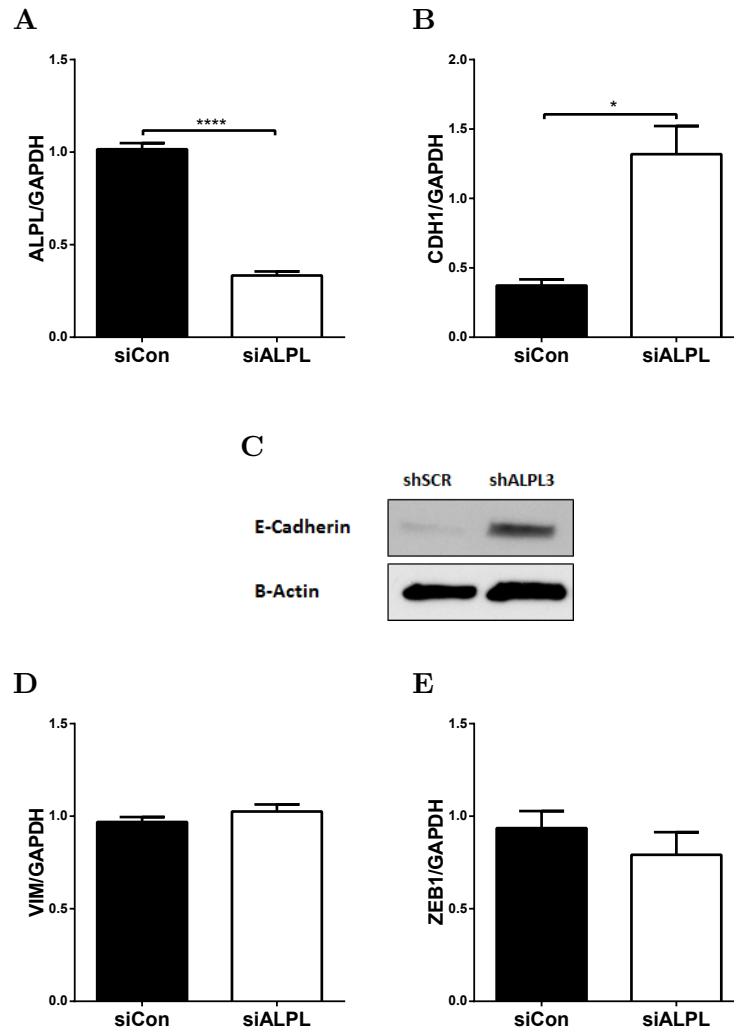


Figure 3.8: Knockdown of ALPL in ARCaPM cells results in increased expression of epithelial marker E-cadherin (A) ALPL-targeting or control siRNA (50 nM) was transfected into ARCaPM cells for 72 hours and ALPL mRNA was measured using SYBR Green real-time RT-PCR. (B) E-cadherin mRNA and protein expression were quantified in ALPL knockdown or control ARCaPM cells using SYBR Green real-time RT-PCR or Western blotting respectively. (C) Vimentin and ZEB1 mRNA expression were measured in ARCaPM cells following ALPL knockdown using SYBR Green real-time RT-PCR. All mRNA expression data were normalized with GAPDH expression. (Data are representative of 3 independent experiments in triplicates and are shown as mean $\pm$ S. E. * $P < 0.05$, **** $P < 0.0001$)

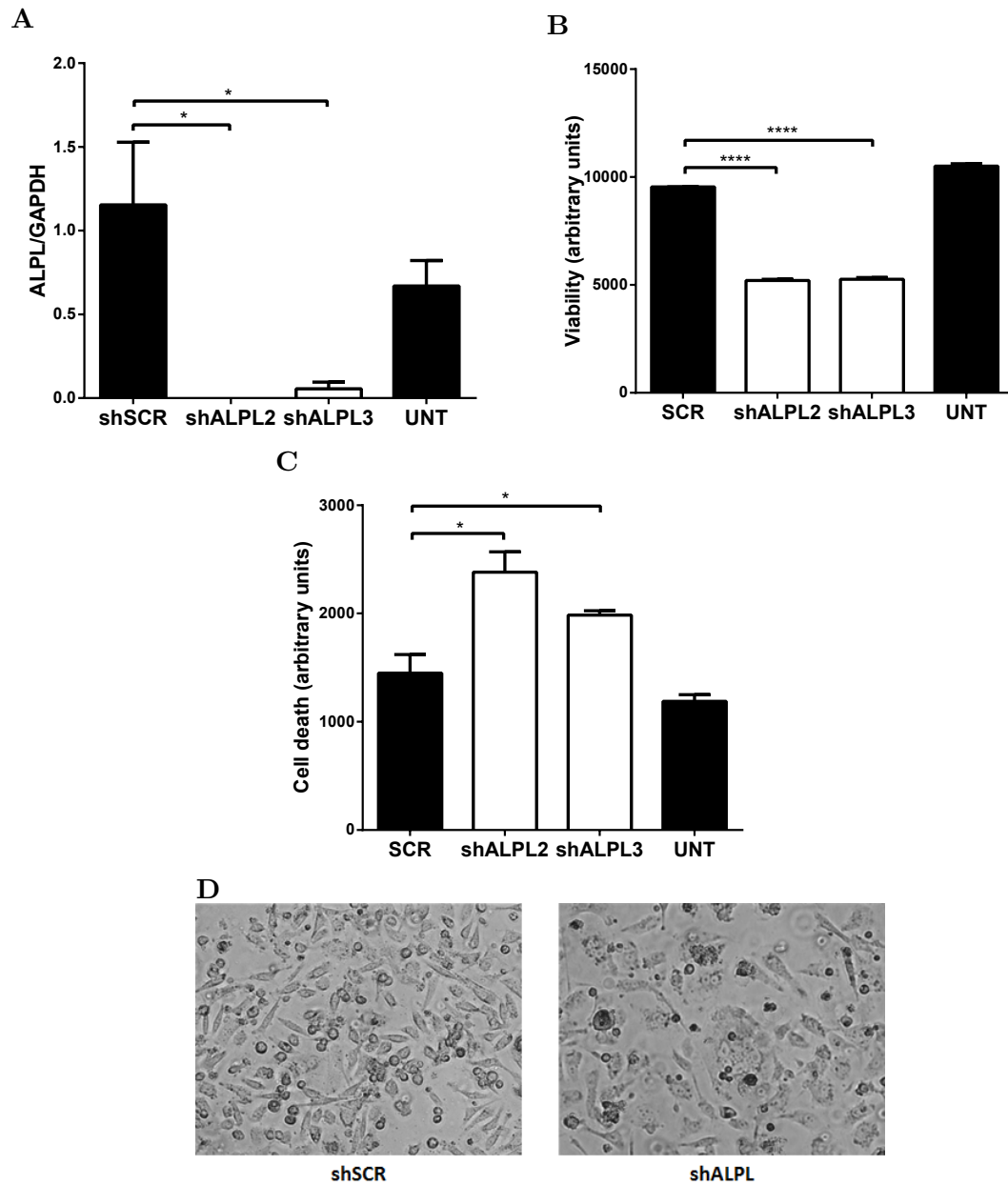


Figure 3.9: Knockdown of ALPL induces change in morphology, reduction in viability and increased cell death in PC3 cells (A) ALPL mRNA was measured in PC3 cells transfected for 72 hours with ALPL-targeting shRNA using real-time RT-PCR. (B) Cell viability was measured using Alamar Blue assay. (C) Cell death was measured using YO-PRO-1 dye staining. D. Cell morphology was imaged using a Nikon inverted microscope at 10X magnification. (Data are representative of 2 independent experiments in triplicates and are shown as mean $\pm$ S. E. * $P < 0.05$, **** $P < 0.0001$)

3.4.9 Inhibition of ALP activity with levamisole decreases cell viability and induces cell death in ARCaPM cells

Levamisole is commonly used as an inhibitor of ALP activity *in vitro* and *in vivo*[202]. Hence, it was used as a tool to pharmacologically inhibit ALP activity in prostate cancer cells and determine its effects on cell viability. Levamisole inhibited the intrinsic alkaline phosphatase activity present in growth medium without cells (containing 10% fetal bovine serum) in a concentration-dependent manner, with ALP activity reaching background levels (equivalent to that in serum-free medium) at 6 mM concentration (Fig. 3.10A). Levamisole decreased viability in ARCaPM cells in a concentration-dependent manner, with little or no effect at 0 - 1 mM concentration and a significant reduction by 40% ($P < 0.0001$) at 6 mM concentration (Fig. 3.10B). A concomitant concentration-dependent increase in cell death was observed in levamisole-treated ARCaPM cells, with a 300% increase at 6 mM concentration (Fig. 3.10C). However, no significant reduction in cell viability or increase in cell death were observed at levamisole concentrations less than 3 mM, whereas there was a significant inhibition of ALP activity. Preliminary experiments with 22Rv1 also showed a similar decrease in cell viability upon levamisole treatment (Fig. A.1).

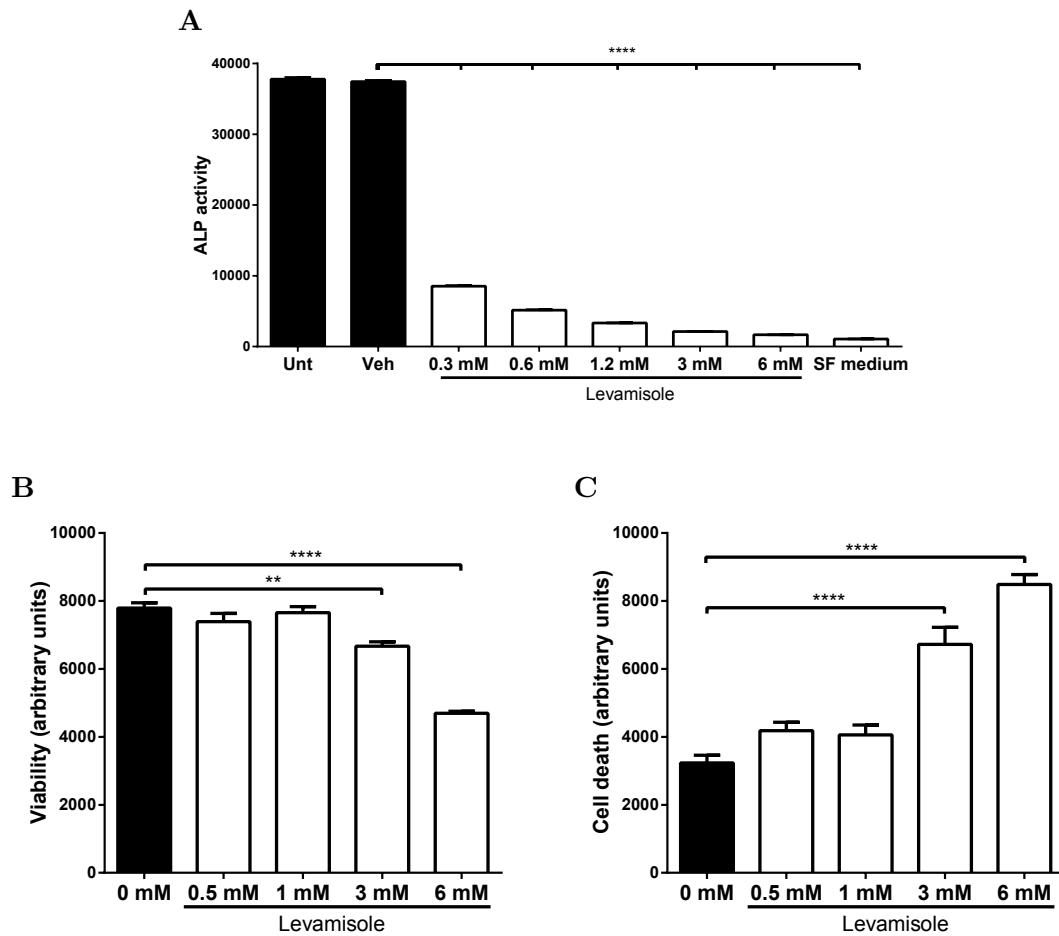


Figure 3.10: Pharmacological inhibition of ALP activity decreases viability and induces cell death in ARCaPM cells in a concentration dependent manner. (A) Intrinsic ALP activity was measured in complete growth medium without cells (containing 10% fetal bovine serum) with increasing concentrations of levamisole using a fluorogenic substrate assay. (B) Cell viability was measured in ARCaPM cells treated with levamisole for 24 hours using Alamar Blue fluorimetric assay. (C) Cell death was measured in ARCaPM cells treated with levamisole for 24 hours using YO-PRO-1 dye. (Data are representative of 3 independent experiments in triplicates and are shown as mean $\pm$ S. E. ** $P < 0.01$, **** $P < 0.0001$)

3.4.10 Snail knockdown in ARCaPM cells results in decreased ALP activity

The results above demonstrate that alkaline phosphatase regulates EMT in ARCaPM cells. Since ARCaPE and ARCaPM cells differ both in terms of EMT and alkaline phosphatase expression, it was aimed to determine the effect of EMT on alkaline phosphatase. Snail (SNAI1) is a key transcription factor that regulates EMT in prostate cancer. Transfection of SNAI1-targeting siRNA resulted in a 40% reduction ($P < 0.05$) (Fig. 3.11A) in Snail mRNA expression compared to control siRNA-transfected ARCaPM cells, measured by real-time RT-PCR. Snail knockdown in ARCaPM cells led to a 20% reduction in ALP enzymatic activity ($P < 0.01$) (Fig. 3.11B). SNAI1 knockdown also resulted in a change from mesenchymal to epithelial morphology, confirming the effect on EMT in ARCaPM cells (Fig. 3.11C).

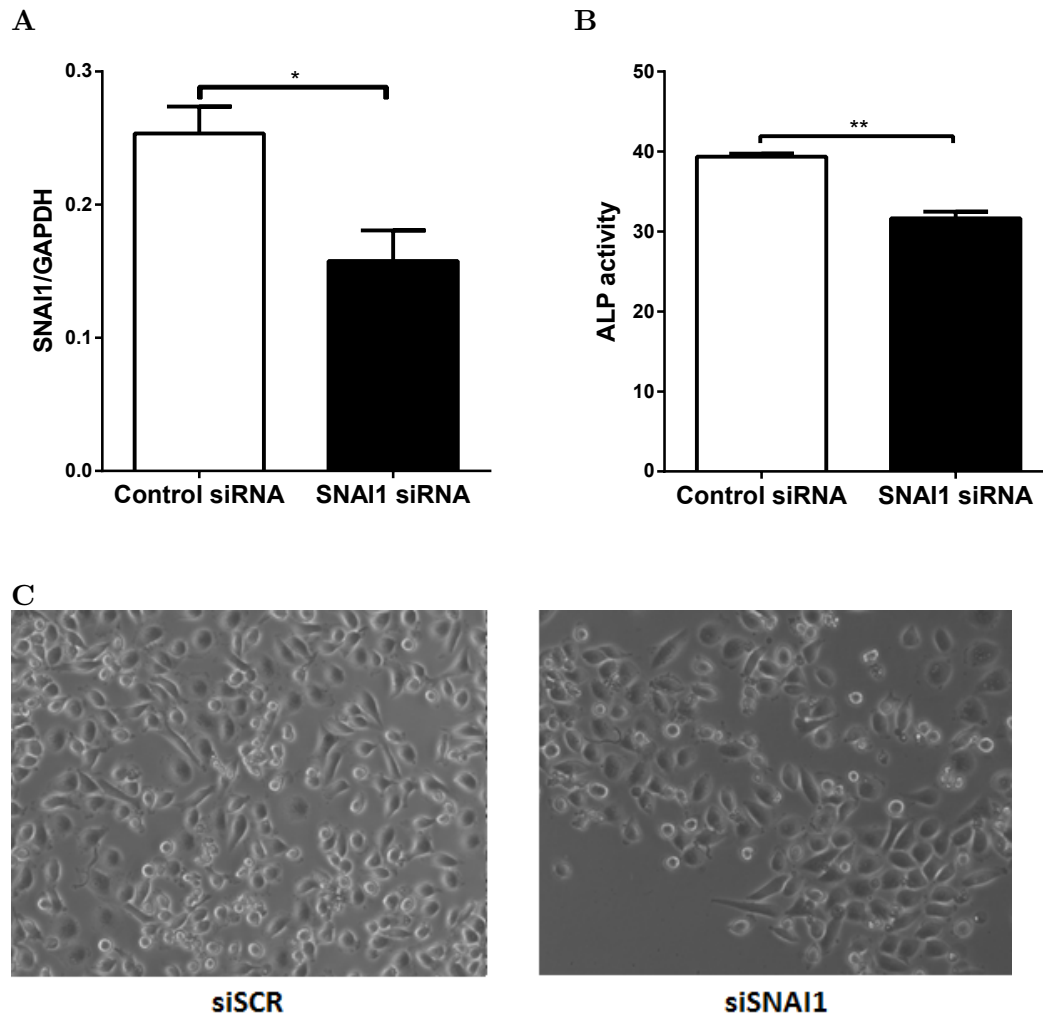


Figure 3.11: Snail knockdown in ARCaPM cells results in decreased ALP activity (A) siRNA-mediated SNAI1 (50 nM for 72 hours) knockdown in ARCaPM cells was confirmed by measuring SNAI1 mRNA expression using SYBR Green real-time RT-PCR and normalizing it with GAPDH expression. (siSCR - scrambled control siRNA) (B) ALP activity in SNAI1 knockdown ARCaPM cell lysates was measured using a fluorogenic substrate assay and normalized to total protein concentration. (C) Microscopic images (10X magnification) of ARCaPM cells transfected with control or SNAI1 siRNA. (Data are representative of 2 independent experiments in triplicates, shown as mean $\pm$ S. E. * $P < 0.05$, ** $P < 0.01$)

3.4.11 Alkaline phosphatase expression correlates positively with Snail and negatively with PTEN expression in patients with prostate cancer

The above results demonstrated an important role for ALPL in two different prostate cancer cell lines, ARCaPM and PC3. However it is important to corroborate such *in vitro* findings in patient samples. Gene expression data from a publicly available dataset Taylor et al.[203] (MSKCC dataset) were mined from cBioPortal[192]. It consists of 150 samples for which complete mRNA expression data are available, of which 131 samples are from primary prostate adenocarcinoma and 19 from bone/liver/lung/lymph node metastases. Correlation of ALPL expression against other genes of interest, which have been implicated in EMT and/or tumour biology, was measured using Spearman's Rank correlation coefficient (ρ , ρ) or Pearson's correlation coefficient (r). High ALPL levels were associated with high SNAIL1 levels, as seen by a moderate positive correlation ($\rho = 0.5827$, $r = 0.5120$) ($P < 0.0001$) (Fig. 3.12A). When the samples were sorted according to their ALPL expression and equally divided into two groups one with lower ALPL expression (designated as "ALPL low") and the other group with higher ALPL expression (designated as "ALPL high"), SNAIL1 expression was significantly higher in "ALPL high" samples ($P < 0.0001$) (Fig. 3.12B). PTEN is a tumour suppressor frequently mutated in prostate cancer. Loss of PTEN is also associated with EMT due to consequent activation of the PI3K/AKT signalling pathway. In the MSKCC dataset, high ALPL expression was associated with low PTEN expression, as seen by a moderate negative correlation ($\rho = -0.5487$, $r = -0.3338$) ($P < 0.0001$) (Fig. 3.12C). Further, PTEN expression in "ALPL high" samples was significantly lower compared to "ALPL low" samples ($P < 0.0001$) (Fig. 3.12D). ALPL expression was weakly negatively correlated with both E-cadherin ($\rho = -0.3614$, $r = -0.3051$) ($P < 0.001$) and ZEB1 expression ($\rho = -0.2888$, $r = -0.2494$) ($P < 0.01$) (Fig. A.2).

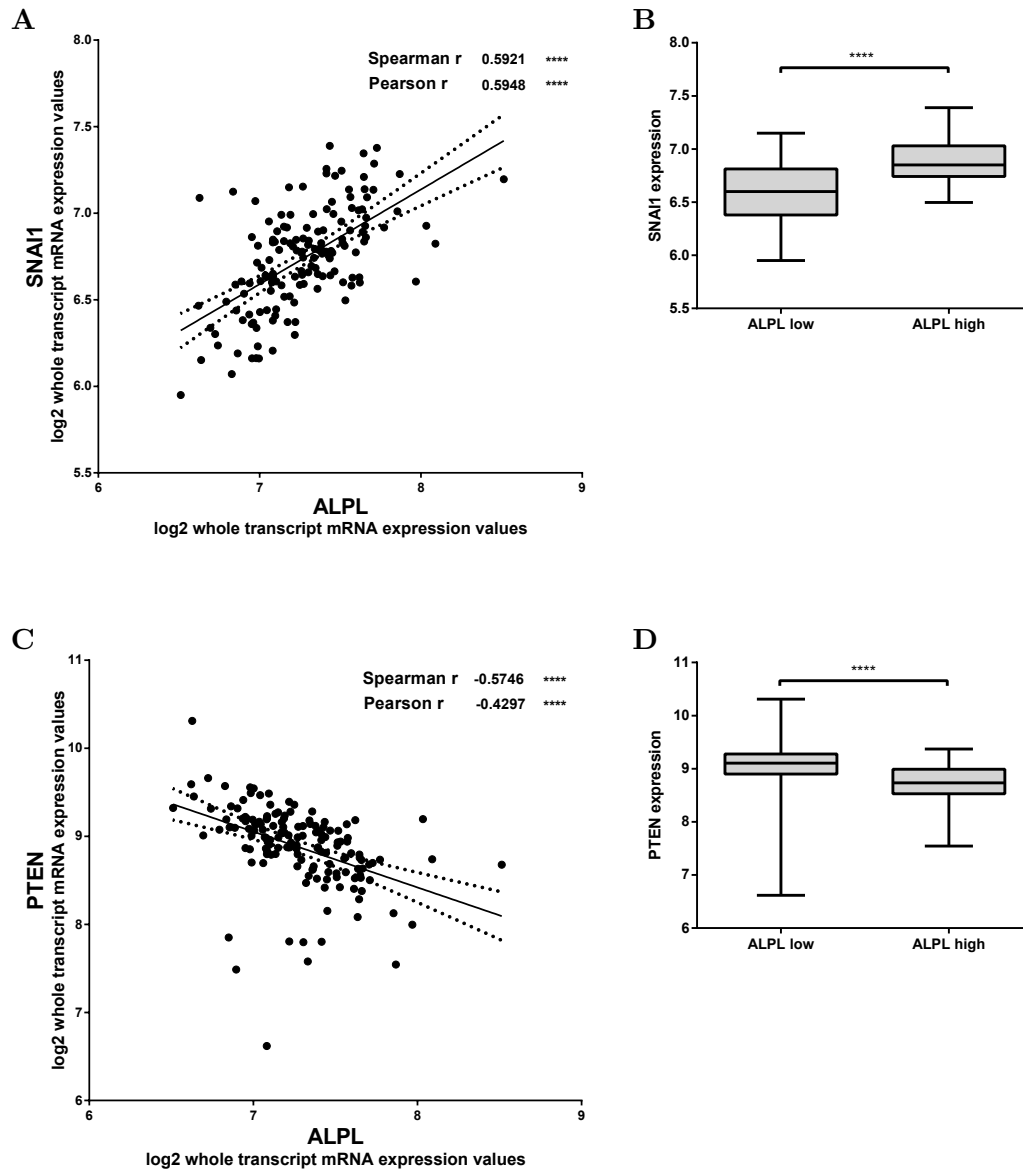


Figure 3.12: ALPL expression is positively correlated with SNAI1 and negatively correlated with PTEN expression in human prostate cancer samples in the MSKCC dataset (A) mRNA expression data was mined from a previously published dataset. ALPL mRNA expression was plotted against SNAI1 expression or (C) PTEN expression. Solid lines represent linear regression and the dotted lines are 95% confidence intervals. (B) SNAI1 or (D) PTEN mRNA expression from ALPL low and ALPL high samples; upper and lower whiskers represent maxima and minima whereas the three horizontal lines of the boxes depict upper, median and lower quartiles. (**** P < 0.0001)

3.5 Summary

These data demonstrate that ARCaPM, 22Rv1, VCaP, DuCaP and PC3 prostate cancer cells express high levels of ALPL mRNA. The isogenic cell lines ARCaPE and ARCaPM exhibit an epithelial or mesenchymal phenotype respectively and differ significantly with respect to alkaline phosphatase: both ALPL mRNA as well as ALP activity are significantly higher in ARCaPM cells compared to ARCaPE. ARCaPM cells can also mineralize *in vitro* while ARCaPE cells do not exhibit this behaviour.

Lentiviral shRNA-mediated knockdown of ALPL is effective in ARCaPM cells, evidenced by a significant decrease in both ALPL mRNA and ALP activity levels. ALPL knockdown using this method in ARCaPM cells resulted in a change from mesenchymal to epithelial morphology. There was a concomitant decrease in migration *in vitro* and a significant decrease in cell viability and increase in cell death. ALPL knockdown in ARCaPM cells resulted in a marked increase in E-cadherin mRNA and protein levels. Similarly, ALPL knockdown in PC3 cells, confirmed by decreased ALPL mRNA expression, resulted in a change from mesenchymal to epithelial morphology, decreased viability and increased cell death.

Knockdown of Snail, an oncogenic EMT transcription factor resulted in a modest decrease in ALP activity in ARCaPM cells. Snail expression was significantly and positively correlated with ALPL expression in human prostate cancer samples in the MSKCC dataset.

3.6 Conclusions

The aim of this study was to determine the role of ALPL in prostate cancer bone metastasis. The results presented here demonstrate that ALPL is a crucial regulator of EMT and cell death in ARCaPM and PC3 prostate cancer cells.

3.6.1 ARCaP model

ARCaPE and ARCaPM cells have been reported to differ in their ability to metastasize to bone in a xenograft mouse model[80][79][63][151]. They also exhibit distinct morphologies *in vitro*: ARCaPE cells are rounded and grow in clusters whereas ARCaPM cells are more elongated and scattered in adherent cell culture. ARCaPM cells exhibit significantly less cell death compared to ARCaPE cells. As has been shown previously by Zhau et al.[80], ARCaPE cells express very high levels of E-cadherin and ARCaPM cells express high levels of vimentin mRNA. This, in conjunction with their *in vitro* morphology, confirms that ARCaPE and ARCaPM cells exhibit epithelial and mesenchymal phenotypes respectively.

3.6.2 *In vitro* mineralization and ALP activity

In vitro mineralization is routinely used as a marker of osteoblast function. Osteogenic medium contains β -glycerophosphate, ascorbic acid and dexamethasone. The alkaline phosphatase secreted by osteoblasts catalyses the cleavage of the phosphate group. The liberated phosphate group in turn combines with free calcium in the growth medium to produce calcium phosphate. This is thought to be a reflection of the mineralization process *in vivo* where alkaline phosphatase similarly catalyses the cleavage of phosphate groups from various substrates like pyridoxal phosphate and ATP, which results in the formation of the inorganic mineral component of bone called hydroxyapatite $Ca_5(PO_4)^3(OH)$. The alkaline phosphatase in osteoblasts is expressed by the ALPL gene and is also called ALP (liver/bone/kidney) or tissue non-specific alkaline phosphatase (TNAP). It is demonstrated here that ARCaPM prostate cancer cells can mineralize *in vitro* whereas the isogenic ARCaPE cells do not mineralize. ARCaPM cells also exhibited a very high basal ALPL expression and ALP activity compared to ARCaPE cells. Other prostate cancer cells have previously been shown to mineralize *in vitro*, including C4-2B, a

derivative of LNCaP. While C4-2B cells did not express a high basal level of ALP, its expression has been shown to increase during prolonged culture in osteogenic medium[190].

It can be speculated that high basal expression of ALP mRNA and activity is associated with the development of osteoblastic bone disease. Intratibial injection of C42B cells in mice results in the formation of osteoblastic bone tumours[204]. Similarly, a subclone of PC3 cells which express high levels of alkaline phosphatase, when injected intratibially in mice, form osteoblastic tumours in contrast to the osteolytic bone tumours typically formed by PC3 cells[205]. Intracardiac injection of ARCaPM cells results in bone tumours of a mixed nature involving both osteolytic and osteoblastic components[145]. Similarly, intracardiac or intratibial injection of 22Rv1 causes osteoblastic bone tumour formation[167]. These reports in conjunction with the present study suggest that osteoblastic bone disease may be associated with high expression of alkaline phosphatase.

3.6.3 Role of ALPL in EMT

Efficient knockdown of ALPL in ARCaPM cells resulted in mesenchymal-epithelial transition (MET) characterised by a change in morphology and a significant increase in E-cadherin mRNA and protein expression. Interestingly, there was no change in the expression of the mesenchymal markers vimentin and ZEB1, suggesting that ALPL-regulated EMT is mainly mediated by E-cadherin. However, the expression of other mesenchymal markers like Snail, Slug, Twist and ZEB2 was not measured in ALPL-knockdown ARCaPM cells; this needs to be determined in future studies. ALPL knockdown also decreased viability and increased cell death ARCaPM, PC3 and 22Rv1 cells. Levamisole, a well known inhibitor of ALP activity, also decreased viability and increased cell death in ARCaPM and PC3 cells, showing that levamisole can be used as a tool to study the role

of ALP *in vitro*. ALPL knockdown resulted in a significantly decreased *in vitro* migration in ARCaPM cells, consistent with mesenchymal-epithelial transition. Taken together, these effects of alkaline phosphatase demonstrate a crucial role for it in prostate cancer cell proliferation and survival as well as their metastatic potential. However, the mechanism through which ALPL regulates these functions is not clear. Known substrates of alkaline phosphatase include the purine nucleotide ATP, pyrophosphate (PPi) and pyridoxal phosphate[206]. It is possible that one or more of these substrates are produced by the prostate cancer cells themselves and can induce MET and cell death; it may then be postulated that high ALP activity reduces the levels of these substrates in the pericellular space, through dephosphorylation. Loss of ALPL expression, and therefore ALP activity, would then result in increased growth-inhibitory substrates, leading to the effects observed upon ALPL knockdown. Pyridoxal phosphate (PLP) is a functionally important molecule that depends on cleavage by ALP activity for transport through the plasma membrane into the intracellular space[49]. However, it was considered unlikely to be a potential mediator of the effects observed in ARCaPM and PC3 cells in *in vitro* cultures because in cell culture media like RPMI, DMEM and T-medium, it is available as a non-phosphorylated pyridoxine form (pyridoxine.HCl) (table B.1) ensuring its transport through the cell membrane without the necessity of dephosphorylation by ALP. As ATP is known to be expressed extracellularly in the tumour microenvironment, is known to induce apoptosis in PC3 cells[207] and since ALPL/TNAP is known to exert functional effects through its ecto-nucleotidase activity[208][209], ATP was considered as a primary candidate to explain the effects of ALPL knockdown. This is further explored in Chapter 4.

Alkaline phosphatase is expressed in promordial germ cells in most mammals, which form the urogenital sinus. Rat prostatic stromal cells express alkaline phosphatase whereas epithelial cells do not express it[210]. This suggests that alkaline

phosphatase may even have a role in the development of the male urogenital system and prostate gland.

Snail is a transcription factor which is important in EMT and is used as a marker of mesenchymal phenotype. Increased expression of Snail induces EMT and conversely, decreasing its expression causes MET. Knockdown of Snail in ARCaPM cells resulted in a change from mesenchymal to epithelial morphology as expected. More interestingly, Snail knockdown resulted in a modest, but significant, decrease in ALP activity. This suggests that alkaline phosphatase itself is regulated by Snail, although it is doubtful whether such a small effect is biologically relevant.

Previously published RNA expression datasets are publicly available for analysis at websites like cBioPortal and ArrayExpress. These represent a wealth of information waiting to be analysed based on specific research questions. In this study, mRNA expression data was obtained from Taylor et al.[203], referred to here as the Memorial Sloan-Kettering Cancer Centre (MSKCC) dataset, using cBioPortal. The analysis identified a moderate positive correlation between the expression of ALPL and SNAI1. While this result on its own must be interpreted with caution, as “correlation does not imply causation”, the finding that Snail regulates ALPL expression in ARCaPM suggests that this regulatory mechanism may not be limited to one particular cell line.

Overall, this study identifies a novel role for bone/liver/kidney alkaline phosphatase in the regulation of epithelial-mesenchymal transition and survival in prostate cancer cells. However, the mechanism of action of alkaline phosphatase in maintaining a mesenchymal phenotype is not clear.

3.6.4 Limitations and further studies

While knockdown of ALPL in this study convincingly demonstrated an important regulatory role for the gene in EMT, the converse experiment was not performed

to check whether overexpression of ALPL results in the induction of EMT in non-cancer prostate cell lines like PNT1a and PNT2 or the epithelial non-metastatic cancer cell line ARCaPE. Further, while levamisole is routinely used as an inhibitor of alkaline phosphatase, more specific inhibitors of TNAP have been discovered recently. Hence, ALP activity inhibition studies need to be confirmed with these novel specific inhibitors.

Any study regarding prostate cancer bone metastasis is incomplete when the cancer cells are studied in isolation; the bone microenvironment needs to be incorporated, either *in vitro* using co-culture systems, or preferably, study the metastatic ability of the cancer cells using *in vivo* mouse models. Xenograft experiments using ALPL knockdown in ARCaPM, PC3 or 22Rv1 and ALPL overexpression in ARCaPE cells would extend the relevance of the *in vitro* findings in this thesis to prostate cancer bone metastasis *in vivo*. If suitable inhibitors become available for *in vivo* use, the effect of blocking ALP activity on tumour uptake and bone metastasis *in vivo* may also be a viable area of focus. Future work will therefore need to address this. In addition to this, the clinical relevance of ALPL as a marker of prostate cancer progression or bone metastasis (apart from its role as a marker of bone turnover) needs to be established. ALPL expression at mRNA and protein levels, and ALP activity measured in primary versus (bone) metastatic samples would answer this to some extent.

‘Have you guessed the riddle yet?’ the Hatter said, turning to Alice again.

‘No, I give it up,’ Alice replied: ‘what’s the answer?’

‘I haven’t the slightest idea,’ said the Hatter.

‘Nor I,’ said the March Hare.

Alice sighed wearily. ‘I think you might do something better with the time,’ she said, ‘than waste it in asking riddles that have no answers.’

—Lewis Carroll, Alice’s Adventures in Wonderland

4

ATP induces mesenchymal-to-epithelial transition in prostate cancer cells

4.1 Introduction

4.1.1 ATP

Cancer cells are highly energetically active. Adenosine triphosphate (ATP), the “energy currency of the cell”, is present at high concentrations intracellularly (3-10 mM), but is thought to be present in low micromolar to millimolar range in extracellular space in the tumour microenvironment. However, direct measurement of extracellular ATP levels is technically challenging and it is unclear what the exact levels might be[211]. Hence, the role of ATP in the growth of cancer cells has been the focus of a number of studies. ATP is a major substrate of ALP and hence it was hypothesized that it may mediate the effects of ALP (Chapter 3). Despite initial scepticism to the hypotheses of Geoffrey Burnstock, it is now well

established that extracellular ATP is involved in cell-cell signalling. ATP acts through a class of mediators called P2X receptors, which in turn are a part of a larger class of receptors called purinergic receptors. Purinergic signalling has been shown to play important roles in a variety of physiological processes including early development, neuronal signalling, immune cell function and musculoskeletal biology[212][213].

4.1.2 Purinergic signalling in cancer

All purinergic receptors are cell surface molecules that respond to purine molecules in the extracellular environment. Purinergic receptors are classified based on their primary agonists: P2X receptors are mainly activated by ATP whereas P2Y receptors are activated by ADP and UTP; A1 and A2 receptors are activated by adenosine. Activation of P2X receptors results in multimer formation. Depending on the receptor and cell type, these multimers may be homomers or heteromers. This leads to the formation of ion channels that allow influx of calcium ions (Ca^{+2}). Increased intracellular (Ca^{+2}) concentration triggers the calcium signalling cascade. In neuronal cells, the ion channels formed by P2X receptors also allow transport of Na^{+} and K^{+} ions, which in turn trigger an action potential resulting in the opening of voltage-gated Ca^{+2} channels[214]. Adenosine receptors, on the other hand, have different structure and mechanism of action from P2X receptors. They are bound to G proteins (also known as GTP-binding proteins) on their intracellular domains leading to inhibition of adenylate cyclase and/or formation of ion channels[215].

4.1.3 Expression and function of P2 receptors in prostate cancer cells

Various purinergic receptors, particularly of the P2X type, are known to be expressed in many cancers. Among these, the P2X7 receptor has been the most studied, but conflicting evidence exists regarding its role in cancer[216]. While it has been shown that P2X7 receptor expression increases tumour growth *in vivo*[217], there is a considerable body of literature showing that these receptors induce apoptosis in cancer cells[218]. The expression of a non-functional P2X7 receptor is up-regulated in prostate cancer[219][220]. Little is known about the role of P2X receptors in prostate cancer. P2X4 increases invasiveness and EMT[221] whereas P2X7 mediates the antitumour effects of Atorvastatin[222] in prostate cancer cells. The Fang group demonstrated that subclones of the PC3 cell line express P2Y2 and extracellular ATP induces *in vitro* invasion through this receptor[223][224][225]. In contrast, the same author has shown that purinergic agonists inhibit prostate cancer cell growth[207] and the Burnstock group has shown that daily administration of ATP inhibits tumour growth in a mouse model of prostate cancer[226].

4.2 Hypothesis and aim

As ATP is one of the substrates of alkaline phosphatase, and knockdown of ALPL caused cell death in ARCaPM and PC3 cells, it was hypothesized that decreased ALP results in increased extracellular levels of ATP and that this may activate purinergic signalling in prostate cancer cells leading to ATP-mediated apoptosis (Fig. 4.1). Hence, it was aimed to test the effects of extracellular ATP on ARCaPM cells, and determine whether it mediates the effects of tumour-derived ALPL.

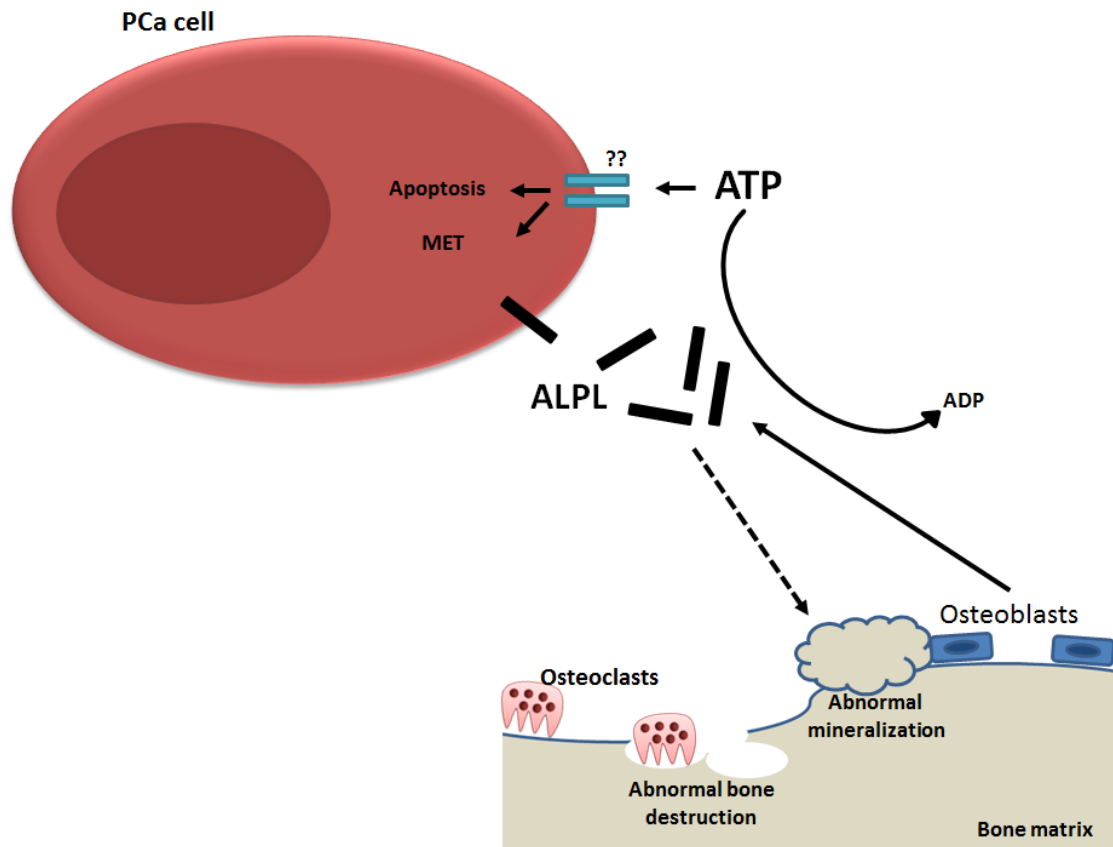


Figure 4.1: Hypothesis Proposed role of ATP as a substrate for the ecto-nucleotidase function of alkaline phosphatase, which degrades ATP and therefore inhibits purinergic signalling in prostate cancer cells

4.3 Methods

4.3.1 High-throughput, automated quantitation of changes in cell shape

Cells seeded in Perkin Elmer CellCarrier-96 plate were stained with Cell Tracker Green CMFDA (Life Technologies, Cat. C2925) (Abs: 492 nm Em: 517 nm), at a final concentration of 1 μ M in serum-free growth medium for 30 minutes. Following this, cells were fixed with 10% formalin for 10 minutes and then stained with DAPI (0.1 mg/ml) (Abs: 358 nm Em: 461 nm) in PBS. The plates were imaged using BD Pathway high content screening microscope. Further analysis of these images was performed on the Attovision software bundled with the BD Pathway microscope. The analysis involved the creation of a mask based on the fluorescence images of the cells and measurement of various shape parameters, including eccentricity ($\propto \frac{1}{circularity}$). From each well of the 96-well plate, at least 2000 discrete cells were analysed and averaged to give the mean eccentricity for each well.

4.3.2 ATP assay

ATP was measured using the ATP bioluminescent somatic cell assay kit (Sigma, Cat. FLASC), based on the luciferin-luciferase reaction. To measure the effect of ALP and apyrase on ATP stability, solutions with or without ATP and the respective enzymes were taken in a 96-well plate and the luciferin-luciferase buffer from the kit was added to the wells using a multi-channel pippette. Luminescence was measured immediately using a luminometer (Fluostar) using an integration time of 1 second.

4.4 Results

4.4.1 Exposure to ATP results in a change in morphology and reduced viability in ARCaPM cells

Although intracellular ATP concentration is in the low millimolar range, the extracellular concentration is thought to be much less, in the micromolar range[211]. Hence, an ATP concentration of 300 μ M was chosen for the following experiments. Treatment of ARCaPM cells with ATP (300 μ M) for 24 hours resulted in a change from elongated, spindle shape to a more rounded morphology (Fig. 4.2A). ATP treatment in serum-free medium for 24 hours also resulted in a significant ($P<0.001$) decrease in viability by 35% whereas ATP treatment in complete growth medium (with 5% FBS) for 24 hours resulted in a significant ($P<0.001$) decrease in viability only by 10% (Fig. 4.2B).

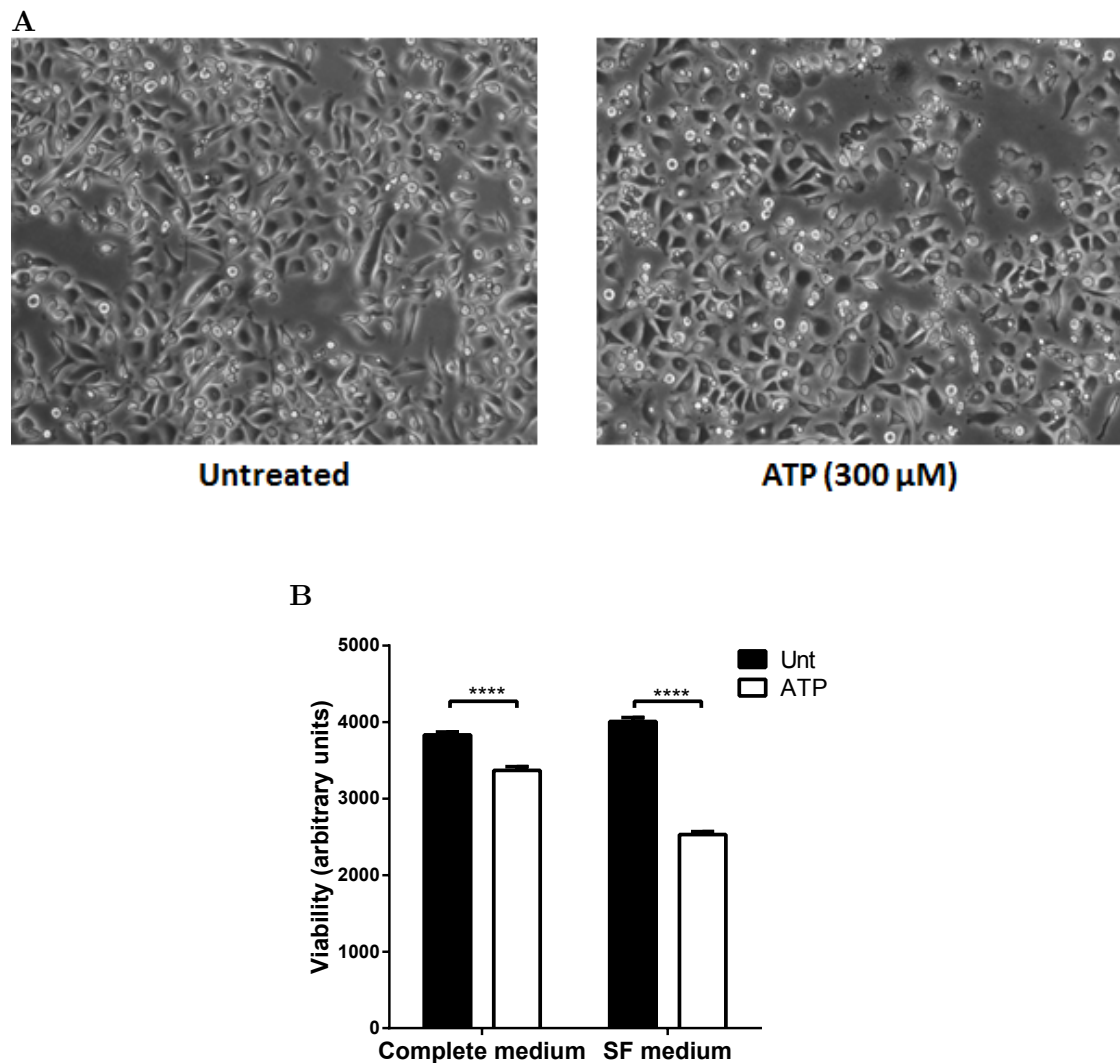


Figure 4.2: ATP induces a change to epithelial morphology and reduces viability in ARCaPM cells (A) Morphology of ARCaPM cells exposed to ATP (300 μ M) (24 hours in complete medium) was imaged using phase contrast microscopy at a magnification of 200X. (B) Viability of ARCaPM cells exposed to ATP (300 μ M) in serum-free or complete growth medium was measured using Alamar Blue fluorimetric assay. (Data are representative of 3 independent experiments and shown as mean $\pm$ S. E. **** P <0.0001)

4.4.2 ATP increases epithelial marker and decreases mesenchymal marker gene expression in ARCaPM cells

Exposure of ARCaPM cells to ATP (300 μ M) for 24 hours in complete medium resulted in a significant increase in the mRNA expression of epithelial markers E-cadherin and cytokeratin 14 by 300% ($P < 0.01$) and 400% ($P < 0.05$) respectively (Fig. 4.3A and 4.3B). A significant increase in E-cadherin protein levels by 70% ($P < 0.05$) was also observed when western blot data was quantitated using densitometry in ImageJ (Fig. 4.3C). In contrast, exposure to ATP resulted in a decrease in the mRNA expression of mesenchymal marker vimentin by 40% ($P < 0.05$) (Fig. 4.3D). The decrease in ZEB1 expression by 40% was not statistically significant (Fig. 4.3E). ATP (300 μ M) treatment of ARCaPM cells for 24 hours in serum-free medium resulted in similar trends of gene expression, with increased mRNA levels of epithelial markers and decreased mesenchymal markers (Fig. 4.3A - 4.3E).

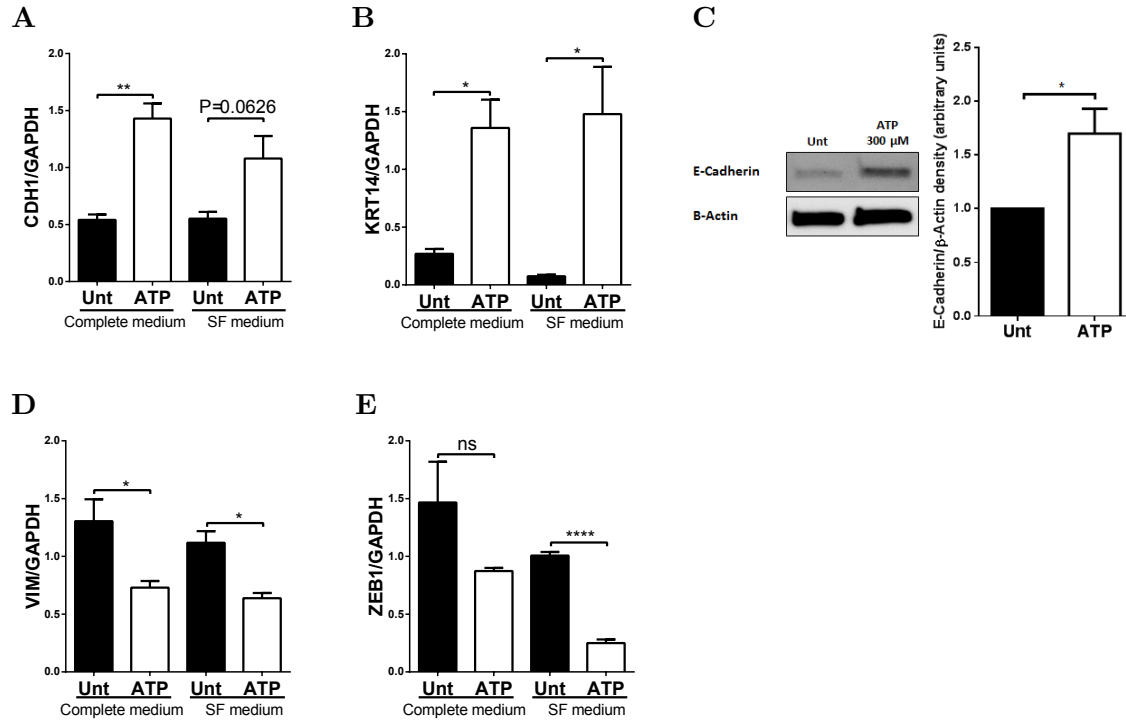


Figure 4.3: Exposure to ATP results in increased expression of epithelial markers and decreased expression of mesenchymal markers in ARCaPM cells (A) E-cadherin, (B) Cytokeratin 14, (D) Vimentin and (E) ZEB1 mRNA expression was measured in control or ATP (300 μ M) treated ARCaPM cells using real-time RT-PCR. GAPDH mRNA expression was used for normalization. (C) E-cadherin protein expression was measured using western blotting and quantified using densitometry on ImageJ software. β -actin protein expression was used for normalization. (Data are from one experiment in triplicates and are shown as mean $\pm$ S. E. ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$)

4.4.3 The effects of ATP are greater than other purinergic agonists in ARCaPM cells

As shown above (Fig. 4.2), ARCaPM cells exposed to ATP (300 μ M) are less viable. Determining the effect of other purinergic agonists like adenosine, ADP and UTP is important in order to dissect the involvement of specific purinergic receptors in the signalling pathway. Exposure of ARCaPM cells to adenosine had no significant effect and exposure to ADP or UTP (300 μ M each) induced a limited decrease in the viability of ARCaPE or ARCaPM cells (Fig. 4.4A and 4.4B).

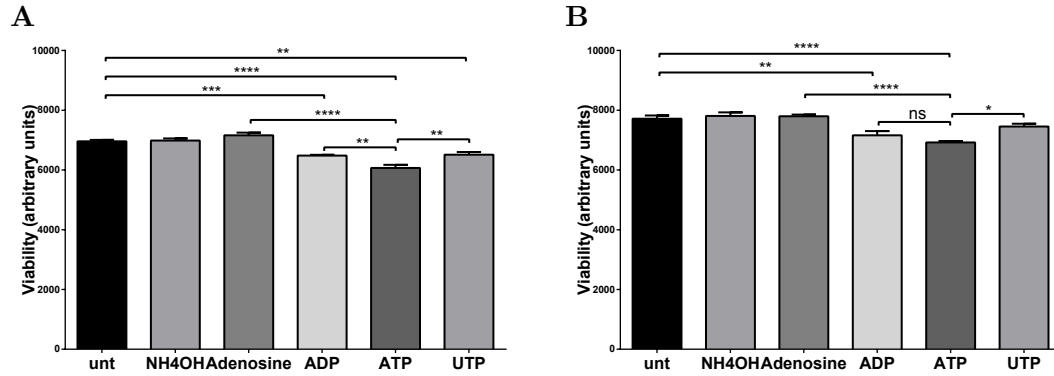


Figure 4.4: Purinergic agonists other than ATP and ADP have little or no effect on cell viability in ARCaPE and ARCaPM cells (A) ARCaPM or (B) ARCaPE cells in complete growth medium were treated with the purinergic agonists adenosine, ADP, ATP and UTP at a concentration of 300 μ M, and viability was measured 24 hours after treatment using Alamar Blue assay. NH4OH is a vehicle control for adenosine. (Data are representative of 3 independent experiments in triplicates and are shown as mean $\pm$ S. E. ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)

4.4.4 High-throughput (automated) quantitation of cell morphology

As change in morphology is a key feature of mesenchymal-epithelial transition, a high-throughput method was deemed useful to quantify the circularity of cells. A preliminary attempt at this was performed using the BD Pathway microscope and high-content analysis system. To begin with, a segmentation mask was created (Fig. 4.5A) of ARCaPE and ARCaPM cells stained with CellTracker Green, a fluorescent dye for staining live cells, and eccentricity of cells was measured using BD Pathway software. Eccentricity of ARCaPM cells was significantly higher ($P < 0.001$) compared to ARCaPE cells (Fig. 4.5B). This technique was used on ARCaPM cells treated with ATP or UTP in combination with the non-specific purinergic antagonists PPADS or Suramin, demonstrating that ATP treatment, alone or in combination with the antagonists, resulted in a significant decrease in eccentricity; however, UTP, PPADS and Suramin treatment, alone or in combination, did not lead to a change in ARCaPM morphology (Fig. 4.5C).

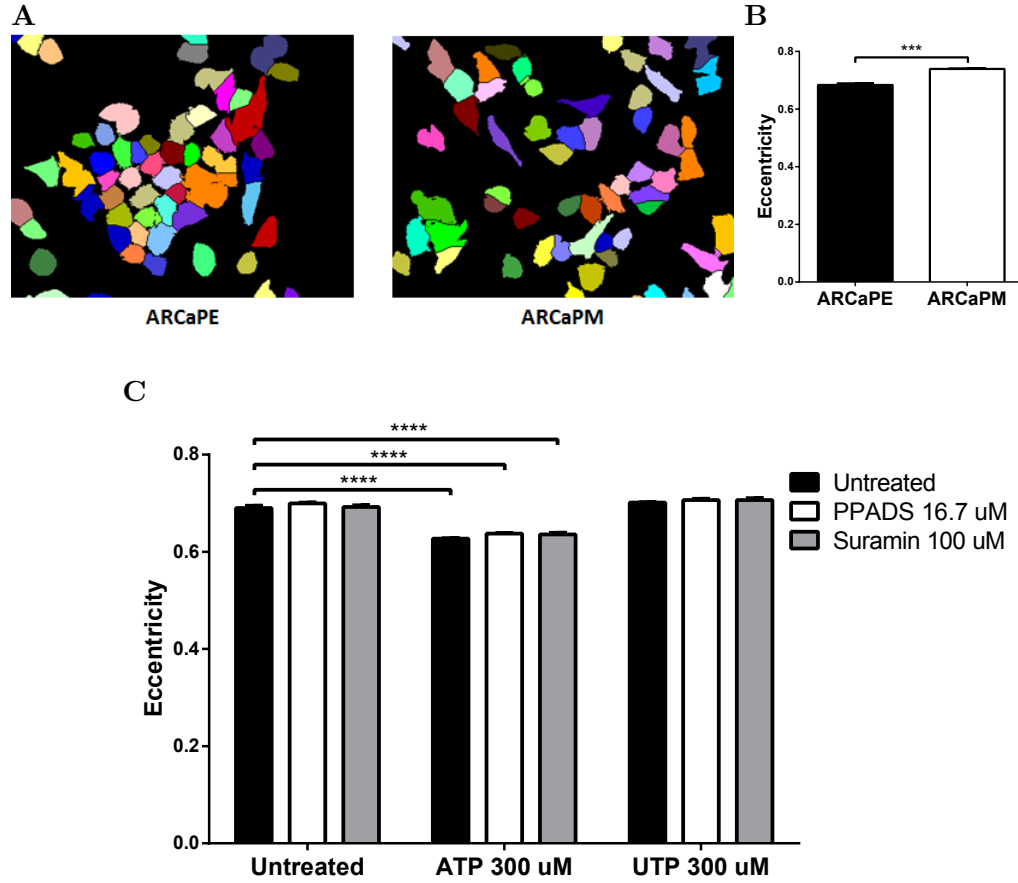


Figure 4.5: Automated quantitation of cell circularity using image acquisition and analysis on the BD Pathway (A) Segmentation mask for ARCaPE and ARCaPM cells stained with Cell Tracker Green (whole cell stain) and DAPI (nuclear stain). (B) Quantitation of eccentricity ($\propto \frac{1}{\text{circularity}}$) in ARCaPE and ARCaPM cells. (C) Quantitation of eccentricity in ARCaPM cells treated with ATP or UTP, with or without PPADS and Suramin. (Means $\pm$ S. E. of data from 3 wells of a 96-well plate shown. ns: *** $P < 0.001$, **** $P < 0.0001$)

4.4.5 Non-specific blockade of purinergic receptors has no effect on ARCaPM cells' response to ATP

If the effect of ATP on ARCaPM cell viability can be rescued by the blockade of purinergic receptors, this indicates that ATP is acting through purinergic receptor signalling. Suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) are non-specific antagonists of purinergic receptors[212]. In ARCaPM cells, PPADS (16.7 μ M) or suramin (100 μ M) in combination with ATP (300 μ M) treatment resulted in a significant decrease in viability by 30% and 40% respectively, which was significantly more than the decrease by 20% seen with ATP (300 μ M) treatment alone. There was no significant change in viability with UTP (300 μ M) alone or in combination with suramin and PPADS (Fig. 4.6).

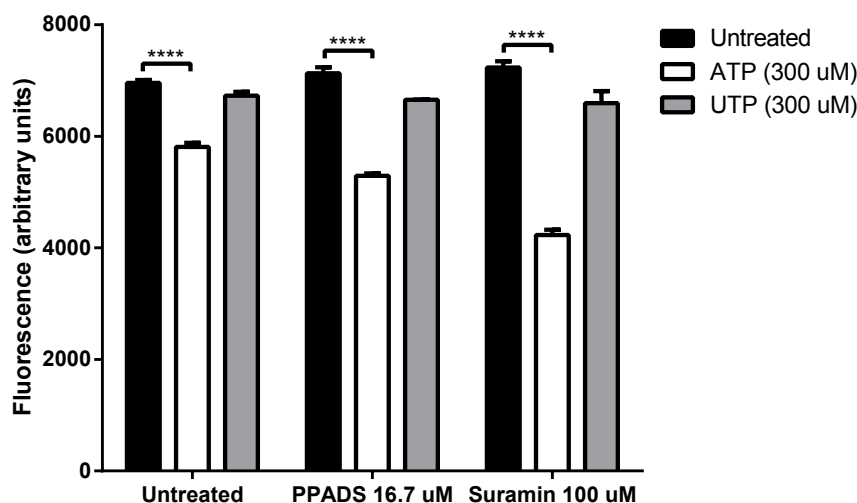


Figure 4.6: Non-specific blockade of purinergic receptors does not abrogate the effects of ATP in ARCaPM cells (Viability was measured in ARCaPM cells in complete growth medium treated for 24 hours with ATP or UTP, alone or in combination with PPADS or suramin, using Alamar Blue assay. (Data are representative of 3 independent experiments in triplicates and are shown as mean $\pm$ S. E. **** P <0.0001)

4.4.6 Expression of the P2X7 purinergic receptor in prostate cancer cell lines

From the above results, it became apparent that certain purinergic receptors (P2Y2, P2Y4, P2Y6, P2Y14) are not likely to be mediating the effects of ATP, due to limited effects of UTP. The activation of the purinergic receptor P2X7 has been most often reported to result in the induction of cell death and apoptosis in cancer cells, in contrast to many other P2X receptors whose activation resulted in increased proliferation and cell survival. Hence, P2RX7 mRNA was measured in a panel of prostate cancer cell lines using SYBR Green real-time RT-PCR (Fig. 4.7). This expression panel demonstrated that P2RX7 is expressed in the majority of the prostate cancer cell lines, albeit at different levels.

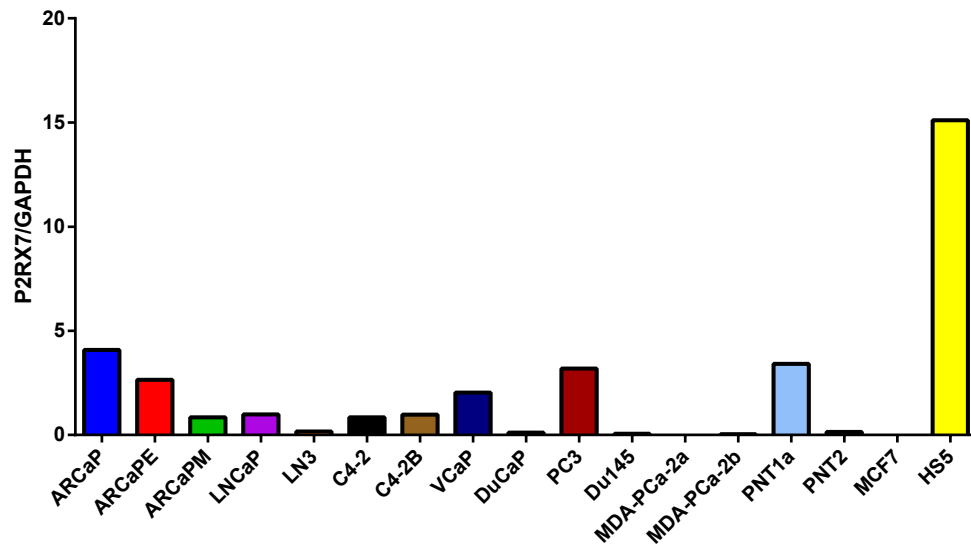


Figure 4.7: The purinergic receptor P2X7 (P2RX7) is differentially expressed in a panel of prostate cancer cell lines P2RX7 mRNA expression in a panel, consisting of non-tumourigenic prostate, non-metastatic prostate cancer, bone-metastatic prostate cancer, prostate cancer cells that metastasize to sites other than bone, and breast cancer cell lines (as described in section 3.4.3), was measured using real-time RT-PCR and normalized with GAPDH expression.

4.4.7 Apyrase does not rescue ARCaPM cells from the effects of ALPL knockdown

As we hypothesised (Fig. 4.1) that ALP exerts its effects on prostate cancer cells through its function as an ecto-nucleotidase, we reasoned that addition of another ecto-nucleotidase could act as a substitute in the absence of ALP. Apyrase is an enzyme derived from plant sources and it efficiently degrades extracellular ATP at physiological pH when added to the growth medium[227]. When ALPL was knocked down by lentiviral transduction in ARCaPM cells, there was a significant reduction in viability compared to control scrambled shRNA. However, there was no difference in viability between untreated cells and cells treated with apyrase(Fig. 4.8). Ecto-nucleotidase function of apyrase was confirmed using an ATP assay (Fig. A.3).

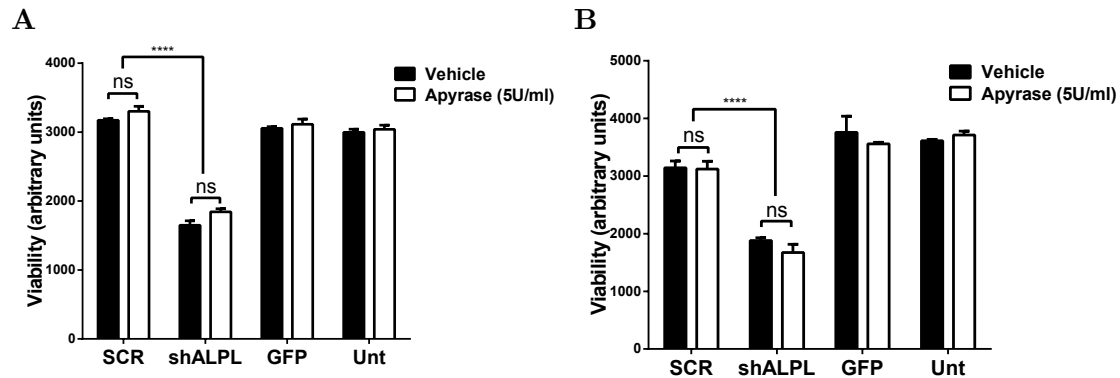


Figure 4.8: The ecto-nucleotidase apyrase does not rescue ARCaPM cells from ALPL knockdown (A) Viability was measured in ARCaPM cells in complete (5% serum) or (B) serum-free medium, transduced with ALPL or control shRNA and simultaneously treated with vehicle or Apyrase (5U/ml) for 72 hours. (Data are representative of 2 experiments in triplicates and are shown as mean $\pm$ S. E. ns - $P > 0.05$, **** $P < 0.0001$)

4.4.8 ATP causes a reduction in alkaline phosphatase expression and activity in ARCaPM cells

Since ATP induced MET, and the mesenchymal state is associated with ALPL expression and activity (Chapter 3), it was hypothesized that ATP may regulate ALP. Exposure of ARCaPM cells to ATP (300 μ M) resulted in a decrease in ALPL mRNA levels by 40% both in complete growth medium ($P < 0.05$) or serum-free conditions ($P < 0.01$) (Fig. 4.9A). There was a concomitant decrease in ALP activity by 35% ($P < 0.001$) (Fig. 4.9B).

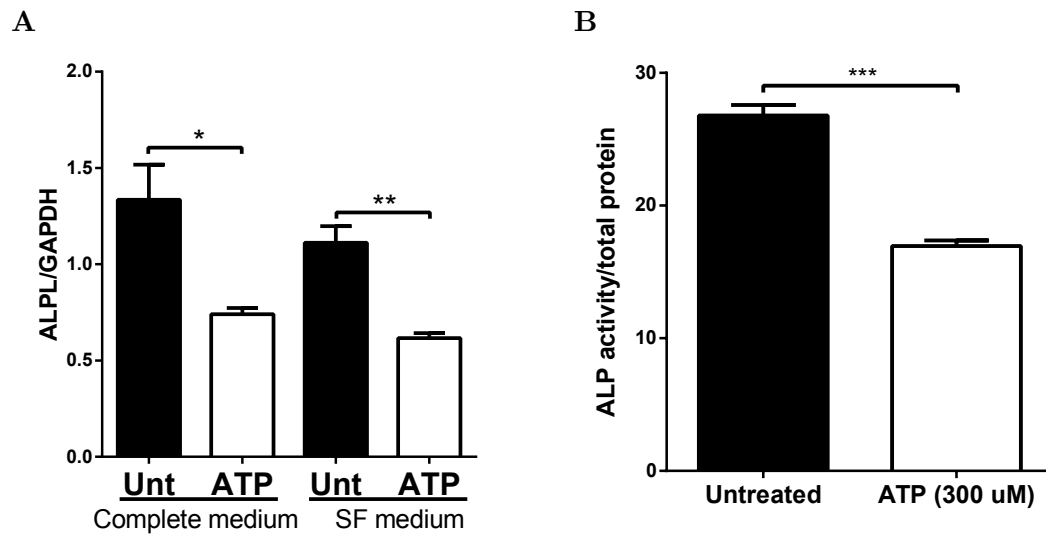


Figure 4.9: Effect of ATP treatment on alkaline phosphatase (A) ALPL mRNA expression in ARCaPM cells treated with ATP (300 μ M) for 24 hours. (B) ALP activity in untreated or ATP-treated ARCaPM cells, measured by a fluorescence assay. (Data are representative of 3 experiments in triplicates and are shown as mean $\pm$ S. E. * $P < 0.05$, * $P < 0.01$, *** $P < 0.001$)

4.5 Summary

Our studies demonstrate that ATP induces a change from an elongated spindle shaped to a more rounded morphology in ARCaPM cells. This was accompanied by a reduction in cell viability, which was of greater magnitude in serum-free conditions. Exposure to ATP also increased the mRNA and protein expression of epithelial marker E-cadherin and mRNA expression of cytokeratin 14, and decreased the mRNA expression of the mesenchymal markers vimentin and ZEB1. Further, ATP treatment of ARCaPM cells resulted in decreased mRNA expression of ALPL and also decreased ALP activity. Comparison of the effects of ATP with other purinergic agonists suggests that ATP causes the greatest reduction in cell viability followed by ADP and UTP. Adenosine had little or no effect on the viability of ARCaPE and ARCaPM cells. The non-specific purinergic antagonists suramin and PPADS had no effect by themselves on the viability of ARCaPM cells whereas in combination with ATP, they had an additive effect in decreasing cell viability. UTP alone or in combination with suramin or PPADS had no significant effect on cell viability in ARCaPM cells.

4.6 Conclusions

Adenosine triphosphate is secreted at high levels by cancer cells. Extracellular addition of ATP has previously been shown to both promote and inhibit growth of cancer cells in various contexts. However, in prostate cancer cells, the effect of ATP is predominantly reported to be growth-inhibitory[228][229][207]. In PC3 cells, previous reports showed evidence for the involvement of P2X5 and P2Y11 receptors in the response to ATP[228]. Our studies with the ARCaPM cell line confirm the growth-inhibitory effects of ATP. It is interesting to note that the effect of ATP is of greater magnitude in serum-free compared to complete growth

medium, suggesting factors that inhibit ATP signalling are present in fetal bovine serum.

4.6.1 Role in MET and involvement of specific purinergic receptors

More importantly, this study shows for the first time the role of ATP in inducing features of mesenchymal-epithelial transition. ATP induces a change to epithelial morphology and an increase in epithelial markers while reducing the expression of mesenchymal markers. Other purinergic agonists adenosine and UTP have no effect on cell morphology or viability whereas ADP decreases ARCaPM cell viability to a lesser extent than ATP. This differential effect of purinergic agonists helps in narrowing down the specific receptors that mediate the effects of ATP. It has been reported that UTP has no effect on some P2Y receptors (P2Y2, P2Y4, P2Y6, P2Y14) ruling these out as mediators of purinergic signalling in ARCaPM cells. Further, adenosine is a strong agonist of the A1 and A2 adenosine receptors and hence, these receptors can be ruled out as mediators of the effects of ATP in ARCaPM cells. While a number of P2X receptors are stimulated by ATP at low concentrations ($<50\mu\text{M}$), only P2X7 is induced by ATP in the high micromolar range[211]. This suggests that P2X7 may mediate ATP signalling in ARCaPM cells. However, an important caveat is that low micromolar concentrations of ATP were not tested on ARCaPM cells and hence the involvement of the other P2X receptors can not yet be ruled out. Previous reports have shown the use of a wide range of concentrations of extracellular ATP[230][231][232][233] and an intermediate concentration was chosen for my experiments. But depending on the specific combination of P2X receptors expressed by ARCaPM cells, their response to different concentrations of ATP may be entirely distinct from those seen with $300\mu\text{M}$. Hence, these experiments need to be repeated with a wide range of lower

ATP concentrations to determine if the same effect can be seen in a concentration-dependent manner.

Use of general and specific-receptor antagonists have been used to elucidate mechanisms of purinergic signalling. The general antagonists suramin and PPADS block a wide range of P2 receptors, depending on the concentration used. The use of suramin or PPADS in combination with ATP should in theory abrogate the effects of ATP on ARCaPM cells. But surprisingly, both Suramin and PPADS potentiated the cytotoxic effects of ATP. One possible explanation for this may be that ATP may be acting on two different receptors in ARCaPM cells, the induction of one leading to cell death and MET whereas the induction of the second receptor promoting proliferation, and suramin or PPADS block the second (pro-survival) receptor to a greater extent than the first (pro-MET) receptor. Further, in discussion with experts in purinergic signalling, it is known that PPADS and suramin are unreliable as purinergic antagonists in some cell types. Hence, future work will address this by making use of antagonists selective to individual receptors. A molecular approach may also be used, to check whether knockdown of specific purinergic receptors would abrogate the effects of extracellular ATP.

4.6.2 ATP and ALP

Importantly, exposure of ARCaPM cells to ATP resulted in decreased alkaline phosphatase expression and activity. While this confirms that the epithelial state in the ARCaP cell line model is associated with low, and a mesenchymal state with high, alkaline phosphatase levels, it also raises the possibility of ALP acting as a mediator of ATP-induced signalling in ARCaPM. This is in contrast to the original hypothesis that ATP mediates the effects of ALPL in MET. The main effects of ATP on ARCaPM cells include induction of MET and decreased cell viability, both of which are remarkably similar to the effects of ALPL knockdown.

However, both these effects are not identical; exposure to ATP caused a decrease in the expression of the mesenchymal markers Vimentin and ZEB1, but there was no significant change in their expression upon ALPL knockdown. This indicates that the effects of ATP on ARCaPM cells may only be partially mediated by alkaline phosphatase, which precludes the possibility of ATP mediating the effects of ALPL knockdown. This is confirmed by the fact that apyrase, of which ATP is a substrate, does not rescue ARCaPM cells from the effects of ALPL knockdown or inhibition (Fig. A.4). Hence, alternative mechanisms need to be explored to explain the effects of ALPL knockdown (chapter 6).

4.6.3 Limitations and future studies

Further work needs to explore the exact mechanism involved in ATP-mediated MET in ARCaPM cells. Use of specific receptor antagonists, e.g. against P2X7, in combination with ATP treatment, may help in narrowing down the potential mediators. Purinergic agonists like Bz-ATP can also be utilised to better characterise these signalling mechanisms. Such agonists, if promising *in vitro*, may also be tested *in vivo* in mouse models, to determine their potential for inhibiting prostate cancer bone metastasis. In addition to receptor agonists and antagonists, knockdown of specific purinergic receptors can be performed to determine the mechanism of action of ATP. Quantitation of cell eccentricity, while demonstrating a statistically significant decrease in ARCaPM cells with ATP treatment, apparently shows proportionally minor differences. This is possibly because of the scale of eccentricity, with an eccentricity = 0 assigned to a perfectly circular object and an eccentricity = 1 assigned to a straight line, both of which are not normally observed in adherent cell lines. However, it should be noted that the difference between ARCaPE vs. ARCaPM is similar to the difference between untreated vs. ATP or UTP vs. ATP. Hence, ARCaPE may be used as positive control cells

for a rounded epithelial morphology and can be used as a baseline against which ATP-treated ARCaPM cells may be compared.

They say a little knowledge is a dangerous thing, but it is not one half so bad as a lot of ignorance.

—Terry Pratchett, *Equal Rites*

5

MicroRNAs regulate EMT and alkaline phosphatase expression in prostate cancer cells

5.1 Introduction

5.1.1 MicroRNAs in prostate cancer bone metastasis

MicroRNAs are 23 nucleotide-long, single stranded non-coding RNA molecules that regulate protein expression by translational inhibition or mRNA degradation[154]. The expression of microRNAs in a cell depends on its developmental stage and tissue type. MicroRNAs are recognized both as biomarkers and therapeutic targets and multiple studies have underlined the importance of microRNAs in PCa causation and progression[157]. Recent studies demonstrated that serum levels of specific microRNAs are altered based on the extent of progression of prostate cancer[159][158][234]. However, few studies have explored the role of microRNAs in the pathophysiology of PCa bone metastasis. miR-203 was

shown to be expressed at lower levels in bone metastatic PCa cell lines and human samples[163]. miR-203 also regulates the Androgen Receptor pathway and Mitogen Activated Protein Kinase (MAPK) pathway in the non-metastatic PCa cells, LNCaP[235]. miR-203 has also been shown to be a key regulator of mesenchymal to epithelial transition (MET) in breast cancer cells[236]. Further, mir-203 has been well established as a key modulator of stemness, primarily by directly targeting P63 in epidermal cells[237][238] and also indirectly by repressing Epithelial to Mesenchymal Transition in pancreatic cancer cells[239]. These findings suggest that miR-203 could have a similar role in repressing stemness in PCa cells.

5.1.2 MicroRNAs regulating EMT

A number of microRNAs have been shown to regulate EMT by targeting various mesenchymal transcription factors. The miR-200 family are the most studied in this respect and are well established as epithelial markers. MiR-200a, -200b and -200c directly repress ZEB1 and ZEB2, thus increasing the expression of E-cadherin, and induce a change from mesenchymal to epithelial phenotype in various cancer cell types, decreasing their invasiveness. Other microRNAs like miR-143 and miR-145 also regulate EMT in prostate cancer cells.

5.2 Hypothesis and aims

Due to microRNAs' known role as potent regulators of various cell signalling pathways, it was hypothesized that they would regulate EMT as well as osteomimicry in prostate cancer cells. The aims of this study were to 1) take a candidate approach, to determine the role of a known bone metastasis-associated microRNA in the regulation of osteomimicry, 2) identify EMT associated microRNAs and 3) develop an unbiased screening technique to identify microRNAs regulating ALP activity.

5.3 Methods

5.3.1 Establishment of miR-203 overexpressing stable cell lines

Plasmids and cloning

The miR-203 precursor (pri-miR-203) was amplified from human genomic DNA using Pfu High Fidelity Polymerase and inserted into pcDNA3.1 (Neo) vector using KpnI and XhoI restriction sites.

Transfection and selection of stable clones

Transfection efficiency of Lipofectamine 2000 in ARCaPE and ARCaPM cells was initially confirmed using a Green Fluorescent Protein-expressing plasmid, pEGFP-N3. MiR-203-expressing or control plasmids were then transfected into ARCaPM cells and the transfected cells were selected for stable expression using the appropriate antibiotic (300ug/ml G418 for the constitutive expression plasmid with Neomycin resistance gene). After 4-6 weeks, the stable pools were grown in maintenance medium, which had selection antibiotics at half the original concentration (150ug/ml G418).

5.3.2 MicroRNA mimic library pilot screening

Cells grown in 96-well plates in triplicates were transfected with 50 nM each of microRNA mimic from plate 5 of the Mission MicroRNA Mimic Library (Sigma), at 90% confluence. 3-6 days following transfection, the conditioned media (growth medium in which the transfected cells were incubated) were collected and ALP activity was measured in these samples as described previously (Chapter 2). Data obtained from the three replicates were averaged for each microRNA and Z-score was calculated using the formula: $Z = (x - \mu)/\sigma$ where x is the mean ALP activity reading for each microRNA, μ is the average of ALP activity of all the

(80) microRNAs (population mean) and σ is the population standard deviation. A Z-score of ± 2 was considered as significant.

5.4 Results

5.4.1 miR-203 inhibits osteomimicry in ARCaPM cells

Since a role for miR-203 in osteolytic PC3 prostate cancer cells has been previously reported[163], this study aimed to determine the effect of miR-203 on osteomimicry by osteoblastic ARCaPM cells and its effect on the expression of ALP. A miR-203 precursor sequence was cloned into a mammalian expression vector pcDNA3.1Neo and stable clones of ARCaPM expressing the control or miR-203 expression plasmids were established by antibiotic selection. Taqman real-time RT-PCR confirmed overexpression of miR-203 by 1000% ($P < 0.001$) compared to empty vector transfected ARCaPM cells (Fig. 5.1A). There was a significant ($P < 0.05$) decrease in both Runx2 and ALPL mRNA expression by 25% in miR-203 overexpressing ARCaPM cells (Fig. 5.1B and 5.1C). Alkaline phosphatase expression in ARCaPM cells is indicative of osteomimicry, characterized by ALP activity and *in vitro* mineralization. miR-203 overexpressing ARCaPM cells show a significantly decreased mineralization in osteogenic medium compared to empty vector transfected cells (Fig. 5.2A) and a significantly ($P < 0.01$) decreased ALP activity by 40% (Fig. 5.2B).

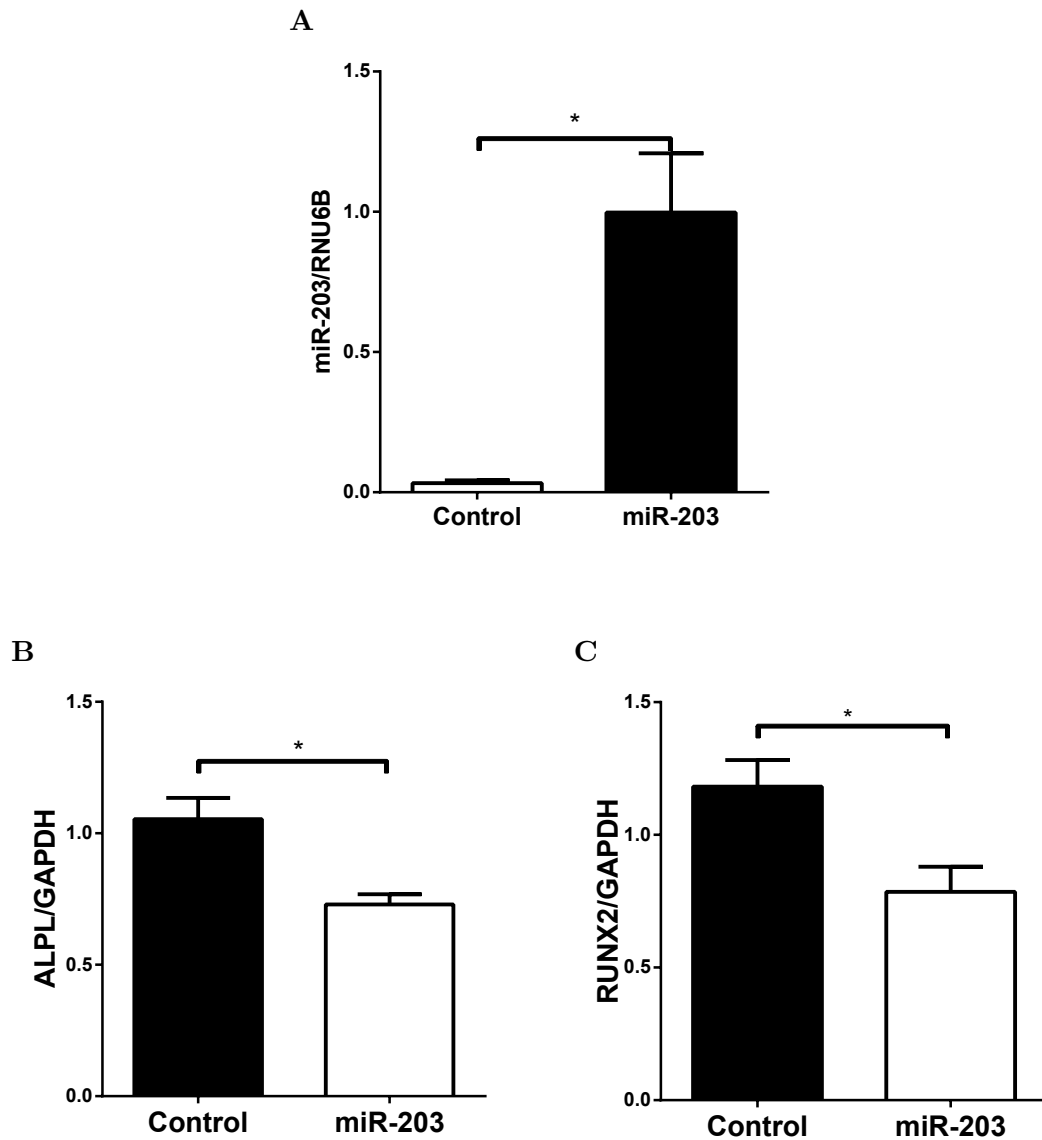


Figure 5.1: Stable expression of miR-203 in ARCaPM cells reduces ALP and RUNX2 mRNA expression. (A) MiR-203 overexpression was confirmed in ARCaPM cells (stable pools) using Taqman real-time RT-PCR. U6 snRNA expression was used for normalization. (B) ALPL and (C) RUNX2 mRNA expression was measured in miR-203 overexpressing or control ARCaPM cells using SYBR Green real-time RT-PCR. Normalization was done with GAPDH expression. (Data are representative of 2 independent experiments in triplicates and are shown as mean $\pm$ S. E. * $P < 0.05$, *** $P < 0.001$)

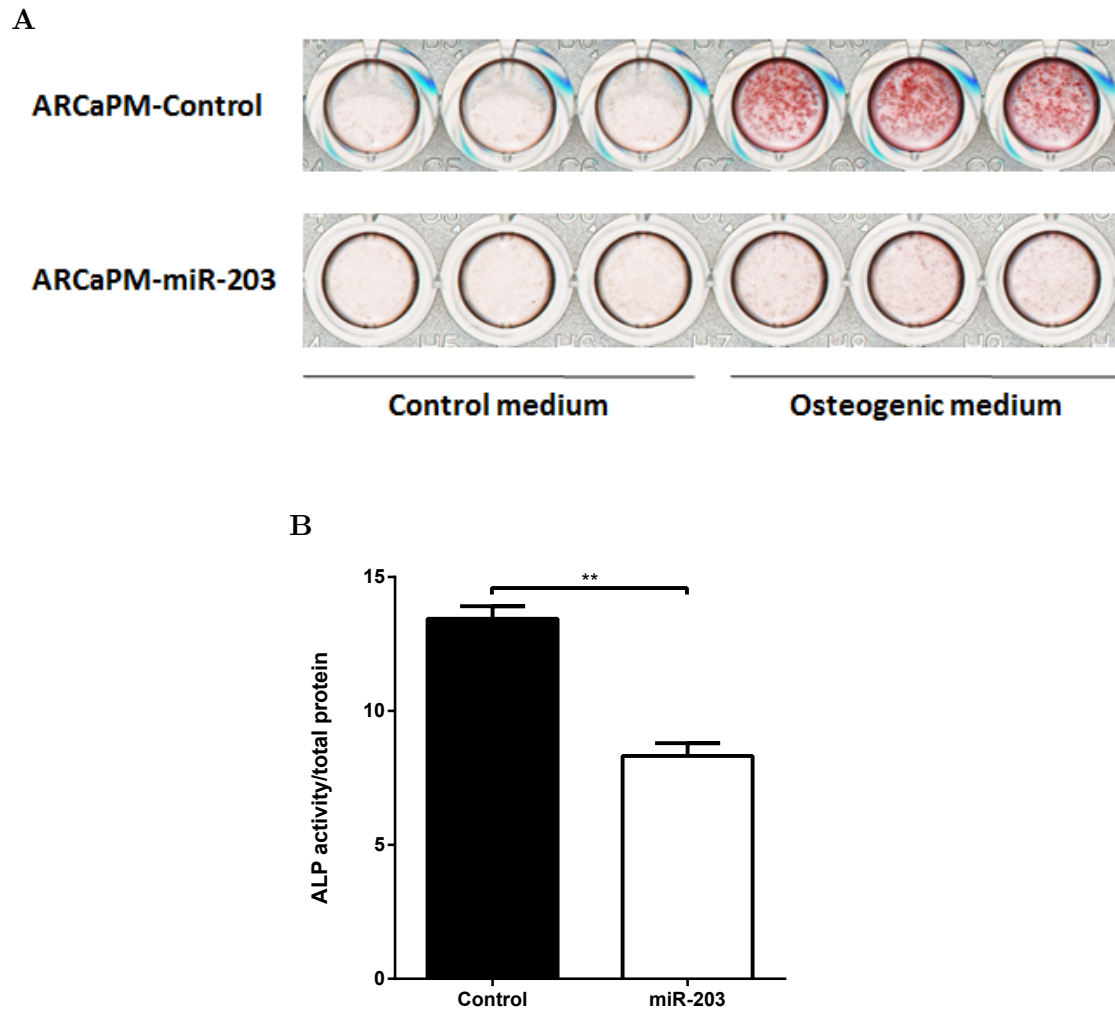


Figure 5.2: Stable expression of miR-203 in ARCaPM cells reduces mineralization and ALP activity. (A) Control or miR-203 overexpressing ARCaPM cells were grown in normal or osteogenic medium for 21 days and stained for mineral deposition using Alizarin Red S. (B) ALP activity in cell lysates was measured using a fluorogenic substrate assay and normalized to total protein concentration. (Data are representative of 2 independent experiments and are shown as mean $\pm$ S. E. ** $P < 0.01$)

5.4.2 Small RNA-sequencing: differential expression of microRNAs between ARCaPE and ARCaPM cells

ARCaPE and ARCaPM cells differ in their ability to metastasize to bone *in vivo* and are a model of epithelial to mesenchymal transition. We hypothesized that microRNAs differentially expressed between ARCaPE and ARCaPM may regulate EMT and therefore mediate metastasis in prostate cancer. To identify differentially expressed microRNAs, total RNA was isolated from ARCaPE and ARCaPM in triplicates, and checked for quality. Following this, small RNA sequencing was performed and quality of the sequencing data was confirmed at the High-Throughput Sequencing Lab, Wellcome Trust Centre for Human Genetics, Oxford. MicroRNAs expressed at a higher level in ARCaPE (Table 5.1) or in ARCaPM (Table 5.2) were sorted based on their logarithmic (base 2) fold change over the other cell line. 57 microRNAs are up-regulated and 68 microRNAs were down-regulated more than 2 fold in ARCaPE compared to ARCaPM cells. Interestingly a distinct co-expression of microRNA clusters was observed in ARCaPE and ARCaPM cells (Fig. 5.3). Members of the miR-371a-372-373, miR-192-194, miR-302abcd, miR-92-363-20b and miR-362-501 clusters were highly expressed ARCaPE cells whereas members of the miR-200ab and miR-181ac clusters were highly expressed in ARCaPM cells (Fig. 5.4). A Bonferroni-corrected P value $<10^{-12}$ was considered as significant.

Table 5.1: RNA-seq: MicroRNAs with higher expression in ARCaPE

High in ARCaPE			
miRNA	logFC (ARCaPM vs ARCaPE)	P Value	False Discovery Rate
hsa-miR-371a-5p	-11.027	6.82×10^{-53}	6.63×10^{-51}
hsa-miR-372	-9.522	4.50×10^{-114}	2.17×10^{-111}
hsa-miR-150-5p	-5.793	8.59×10^{-43}	6.96×10^{-41}
hsa-miR-675-5p	-5.241	1.02×10^{-34}	3.83×10^{-33}
hsa-miR-194-3p	-4.512	5.98×10^{-35}	2.42×10^{-33}
hsa-miR-194-5p	-3.939	1.63×10^{-31}	4.94×10^{-30}
hsa-miR-192-5p	-3.613	6.17×10^{-41}	3.75×10^{-39}
hsa-miR-200c-3p	-2.630	3.25×10^{-19}	4.78×10^{-18}
hsa-miR-629-5p	-2.033	4.31×10^{-13}	4.27×10^{-12}
hsa-miR-148a-3p	-1.959	6.55×10^{-15}	7.23×10^{-14}
hsa-miR-148a-5p	-1.916	1.82×10^{-12}	1.70×10^{-11}
hsa-miR-20b-5p	-1.912	8.89×10^{-10}	6.54×10^{-9}
hsa-miR-4521	-1.859	9.54×10^{-5}	0.000 392 761
hsa-miR-363-3p	-1.854	9.52×10^{-11}	8.41×10^{-10}
hsa-miR-365a-5p	-1.815	3.38×10^{-9}	2.42×10^{-8}
hsa-miR-335-5p	-1.659	2.46×10^{-9}	1.78×10^{-8}
hsa-miR-135a-3p	-1.603	6.12×10^{-9}	4.19×10^{-8}
hsa-miR-449c-5p	-1.588	7.57×10^{-5}	0.000 314 241
hsa-miR-425-5p	-1.547	1.61×10^{-5}	7.67×10^{-5}
hsa-miR-484	-1.517	2.29×10^{-6}	1.24×10^{-5}

P Value shown here is uncorrected.

False Discovery Rate is the expected proportion of false discoveries in all the rejected hypotheses[240].

Table 5.2: RNA-seq: MicroRNAs with higher expression in ARCaPM

High in ARCaPM			
miRNA	logFC (ARCaPM vs ARCaPE)	P Value	False Discovery Rate
hsa-miR-582-3p	7.094	5.59×10^{-80}	9.05×10^{-78}
hsa-miR-100-5p	6.723	1.30×10^{-104}	3.18×10^{-102}
hsa-miR-125b-1- 3p	6.474	3.96×10^{-65}	4.81×10^{-63}
hsa-miR-582-5p	5.938	2.56×10^{-42}	1.78×10^{-40}
hsa-miR-1247-3p	5.570	1.26×10^{-37}	6.14×10^{-36}
hsa-miR-379-5p	5.155	1.94×10^{-40}	1.05×10^{-38}
hsa-miR-429	3.768	1.06×10^{-29}	2.71×10^{-28}
hsa-miR-200a-5p	3.682	2.94×10^{-34}	1.02×10^{-32}
hsa-miR-200a-3p	3.478	8.29×10^{-34}	2.69×10^{-32}
hsa-miR-409-3p	3.218	7.20×10^{-25}	1.46×10^{-23}
hsa-miR-1247-5p	3.164	1.64×10^{-25}	3.62×10^{-24}
hsa-miR-126-5p	3.013	1.18×10^{-17}	1.55×10^{-16}
hsa-miR-147b	2.936	3.65×10^{-21}	5.72×10^{-20}
hsa-miR-200b-3p	2.901	2.22×10^{-28}	5.40×10^{-27}
hsa-miR-381	2.757	6.08×10^{-22}	1.06×10^{-20}
hsa-miR-1	2.486	3.05×10^{-17}	3.80×10^{-16}
hsa-miR-146a-5p	2.340	3.43×10^{-20}	5.21×10^{-19}
hsa-miR-126-3p	2.321	1.78×10^{-16}	2.11×10^{-15}
hsa-miR-95	2.131	2.83×10^{-10}	2.41×10^{-9}
hsa-miR-146a-3p	2.093	8.70×10^{-13}	8.46×10^{-12}

P Value shown here is uncorrected.

False Discovery Rate is the expected proportion of false discoveries in all the rejected hypotheses[240].

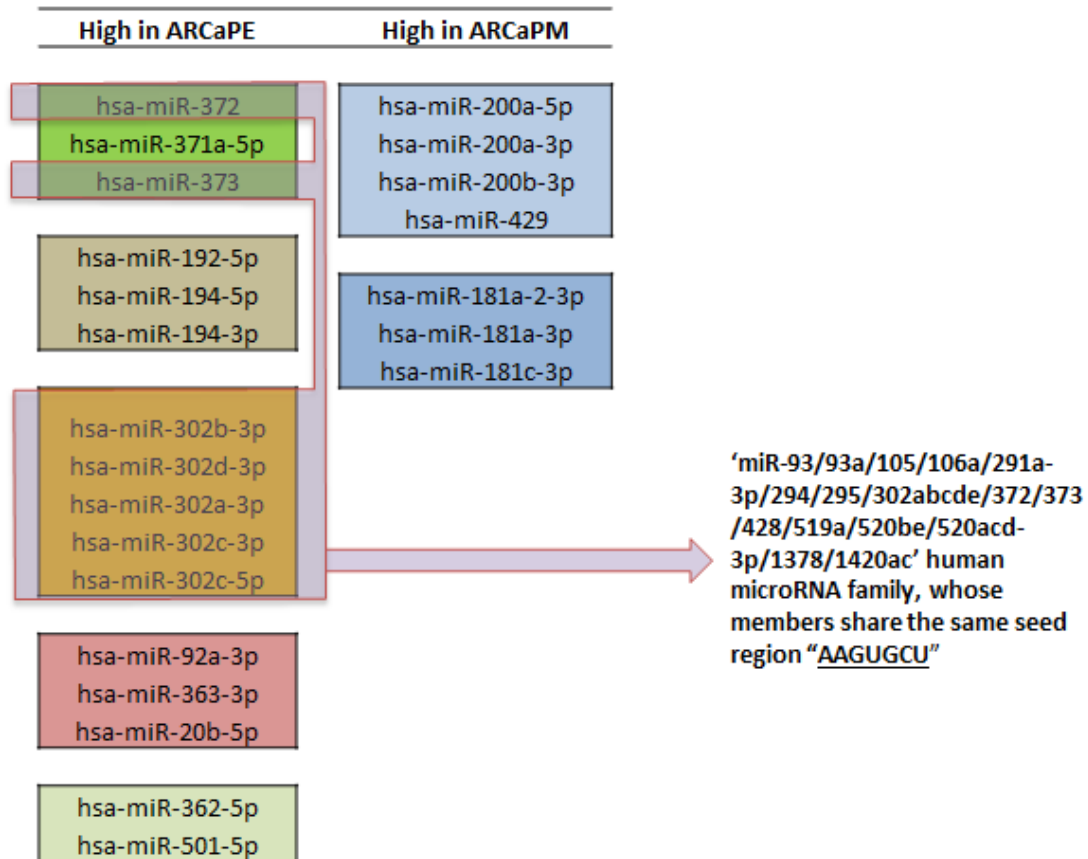


Figure 5.3: miR clusters and families differentially expressed between ARCaPE and ARCaPM MicroRNAs that are expressed from the same genomic locus are collectively called a cluster; each microRNA cluster is depicted in a different colour. MicroRNAs that share a common seed sequence are classified as a family.

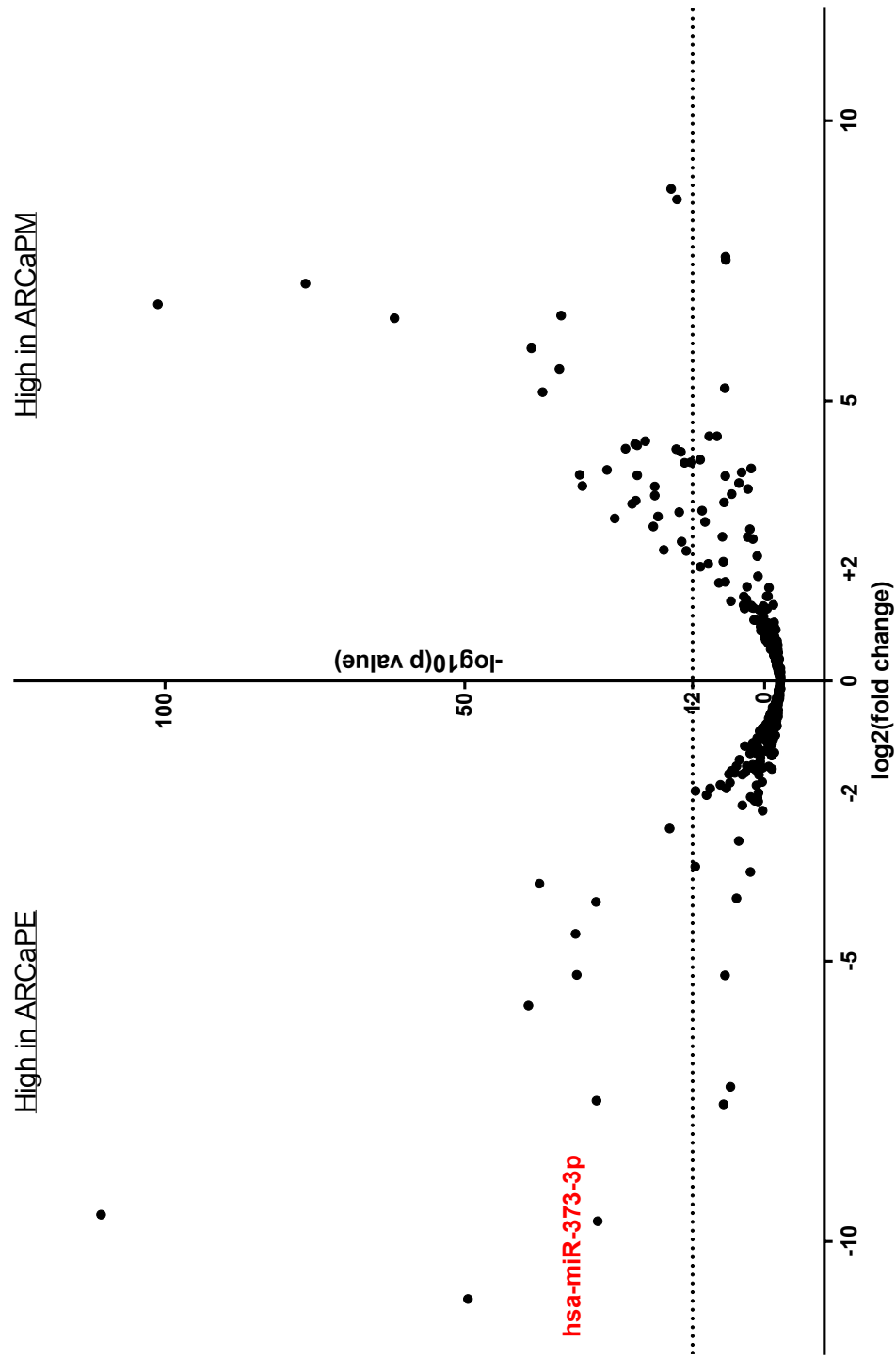


Figure 5.4: Significantly differentially expressed microRNAs. Analysed data from small RNA-sequencing is depicted as a volcano plot. A Bonferroni-corrected p value of $<10^{-12}$ was considered as significant. MiR-373-3p, one of the microRNAs with a highly significant and a large magnitude of fold change, was selected for further study.

5.4.3 Validation of RNA-seq: miR-373 is differentially expressed in ARCaPE vs ARCaPM and induces a mesenchymal-epithelial transition

From a volcano plot of the small RNA-sequencing data (Fig 5.4), it was clear that members of the miR-371-372-373 family were both highly and significantly overexpressed in ARCaPE cells compared to ARCaPM cells. Patrick Kratschmer (an FHS student in Dr. Claire Edwards' lab, who worked under my supervision) confirmed that the expression of miR-373 was significantly higher ($P < 0.05$) in ARCaPE compared to ARCaPM cells (Fig. 5.5).

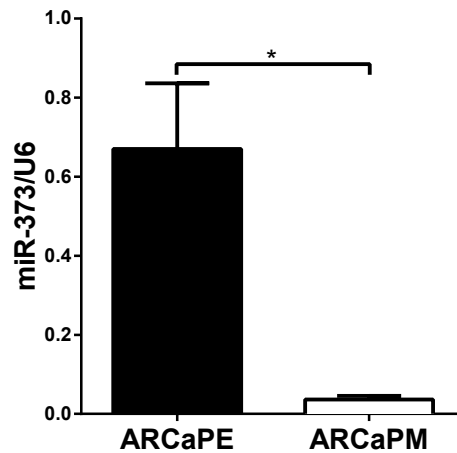


Figure 5.5: Validation of miR-373 differential expression in ARCaPE vs. ARCaPM Differential expression of miR-373 was validated in ARCaPE and ARCaPM cells using Taqman real-time RT-PCR. MiR-373 expression was normalized to RNU6B expression. (Patrick Kratschmer et al., unpublished)(Data are shown as mean $\pm$ S. E and representative of 3 independent experiments. * $P < 0.05$)

However, the functional consequences (if any) of such differential expression needed to be determined. Hence, miR-373 or control mimics were transfected into ARCaPM cells and overexpression of the microRNA, from undetectable to readily-detectable levels ($P < 0.01$), was confirmed using Taqman real-time RT-PCR (Fig 5.6A). Transfection of miR-373 mimic (50 nM) in ARCaPM cells resulted in a change from mesenchymal to epithelial morphology, as observed under phase contrast microscopy (Fig 5.6B). MiR-373 overexpression also resulted in a more than 10-fold increase in the expression of the epithelial marker E-cadherin ($P < 0.001$) (Fig 5.6C), 40% ($P < 0.01$) and 60% ($P < 0.01$) decrease in Vimentin (Fig 5.6D) and ZEB1 expression (Fig 5.6E) respectively in ARCaPM cells, measured by real-time RT-PCR.

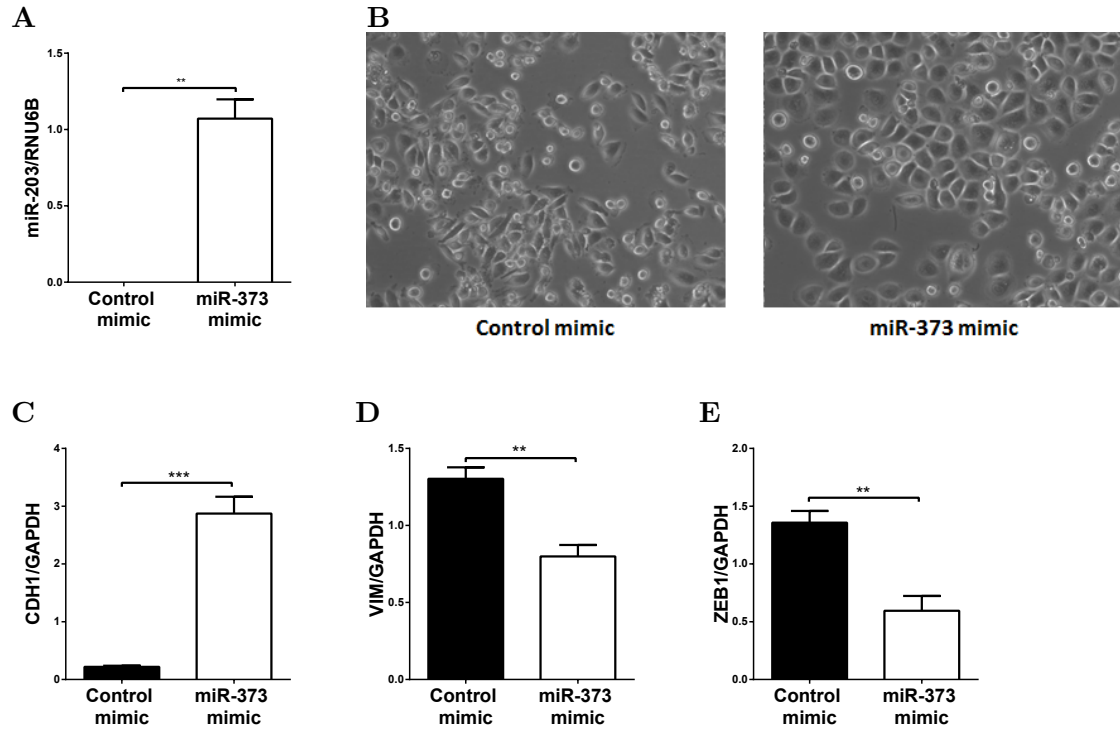


Figure 5.6: MiR-373 overexpression induces mesenchymal-epithelial transition in ARCaPM cells. (A) MiR-373 overexpression (50 nM mimic transfection) was confirmed in microRNA mimic transfected ARCaPM cells using Taqman real-time RT-PCR. (B) Morphology was documented using phase contrast microscopy at 10X magnification (C) E-cadherin, (D) Vimentin and (E) ZEB1 mRNA expression was measured in mimic transfected ARCaPM cells using SYBR Green real-time RT-PCR, with GAPDH expression for normalization. (Data are representative of 3 independent experiments in triplicates and are shown as mean $\pm$ S. E. ** $P < 0.01$, *** $P < 0.001$)

5.4.4 Functional screening of a microRNA mimic library for ALP activity: pilot study identified microRNAs that regulate ALP activity

As ALP expression and activity were associated with EMT in ARCaPM cells (chapter 3) and knockdown of ALPL induced MET and cell death in ARCaPM and PC3 cells, an unbiased high-throughput screen was deemed to be useful in identifying novel microRNAs that regulate ALP activity and expression, and consequently, EMT, in prostate cancer cells. With this aim, a subset (plate 5) of the Mission microRNA mimic library (Sigma) containing 80 microRNAs was transfected in a 96-well format into ARCaPM cells and the secreted ALP activity was measured in the conditioned medium using a fluorescence assay. Initial experiments did not produce consistent results (Fig. 5.7) and the method was optimised extensively, with respect to duration since transfection, frequency of medium change (Fig. A.6) and evaporation effects on the edge of the plate which are some of the issues known to crop up in high-throughput studies of this nature[241]. Z-scores were analysed from the resultant raw data and those microRNAs that fell outside a Z-score of ± 2 were considered to be significant. This identified miR-199a-5p (which came up twice) and miR-212 as potential candidates that regulate ALP activity in ARCaPM cells (Fig. 5.8).

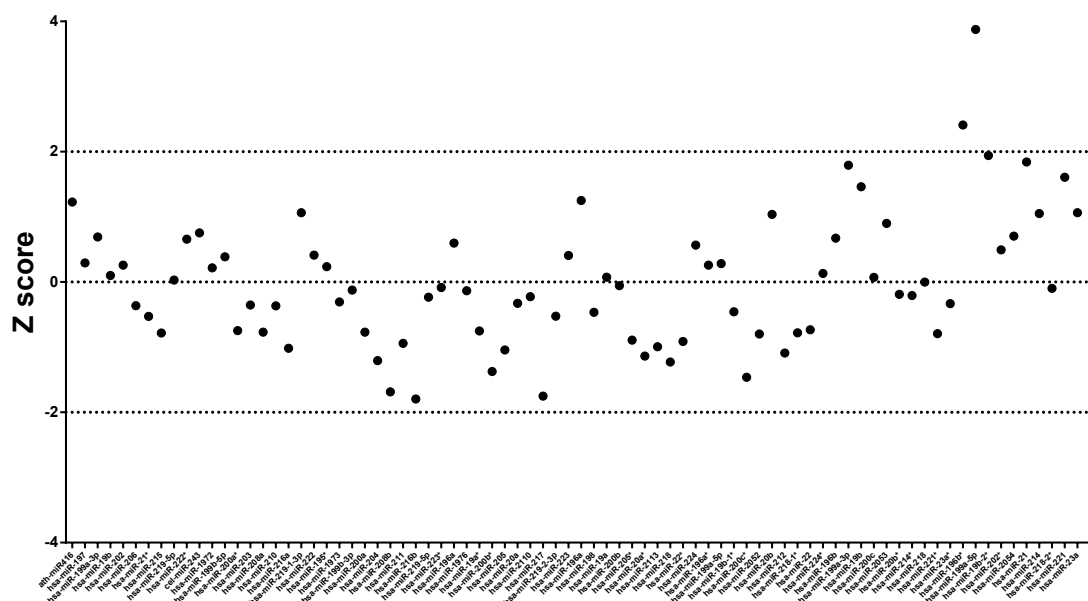


Figure 5.7: ALP activity screen of microRNA mimic library before optimisation MicroRNA mimics from Plate 5 of the Mission MicroRNA mimic Library (SIGMA) were transfected (50 nM each) into ARCaPM cells in a 96-well format and secreted ALP activity was measured in the conditioned media using 4-MUP as fluorescent substrate. Conditioned medium was collected 3 days following transfection.

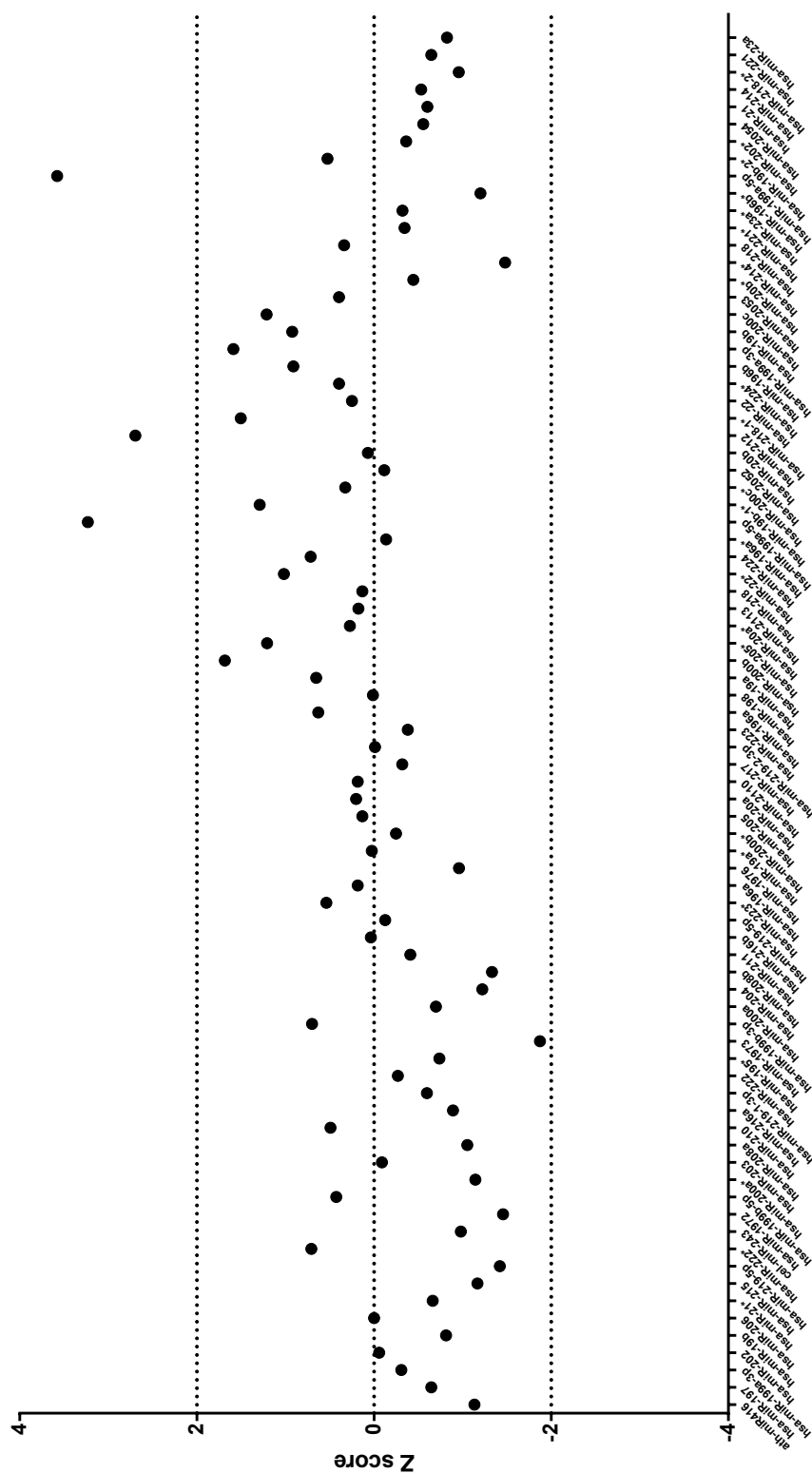


Figure 5.8: ALP activity-based screening of a miR mimic library transfected in ARCaPM cells

MicroRNA mimics from Plate 5 of the Mission MicroRNA mimic Library (SIGMA) were transfected (50 nM each) into ARCaPM cells in a 96-well format and secreted ALP activity was measured in the conditioned media using 4-MUP as fluorescent substrate. Medium was changed 3 days following transfection and conditioned medium for the assay was collected 6 days following transfection.

5.4.5 miR-199a-5p upregulates ALP activity in ARCaPM cells

One of the microRNAs identified in the high-throughput ALP activity screen was selected for further validation. While the mimic library screens were performed using conditioned medium to measure secreted ALP activity, in the validation phase, ALP activity was measured in cell lysates and normalization with total protein concentration. This would eliminate any false hits due to changes in cell number. Transfection of the miR-199a-5p mimic (50 nM) into ARCaPM cells resulted in a significantly increased ALP activity, by 20% ($P < 0.0001$), as measured by a fluorescence assay 72 hours after transfection (Fig. 5.9A). However, there was no significant change in cell viability at the same time point, as measured by an Alamar Blue assay (Fig. 5.9B). Transfection of the miR-199a-5p mimic in ARCaPE cells did not result in either a change in ALP activity or cell viability (Fig. 5.9C)(Fig. 5.9D)

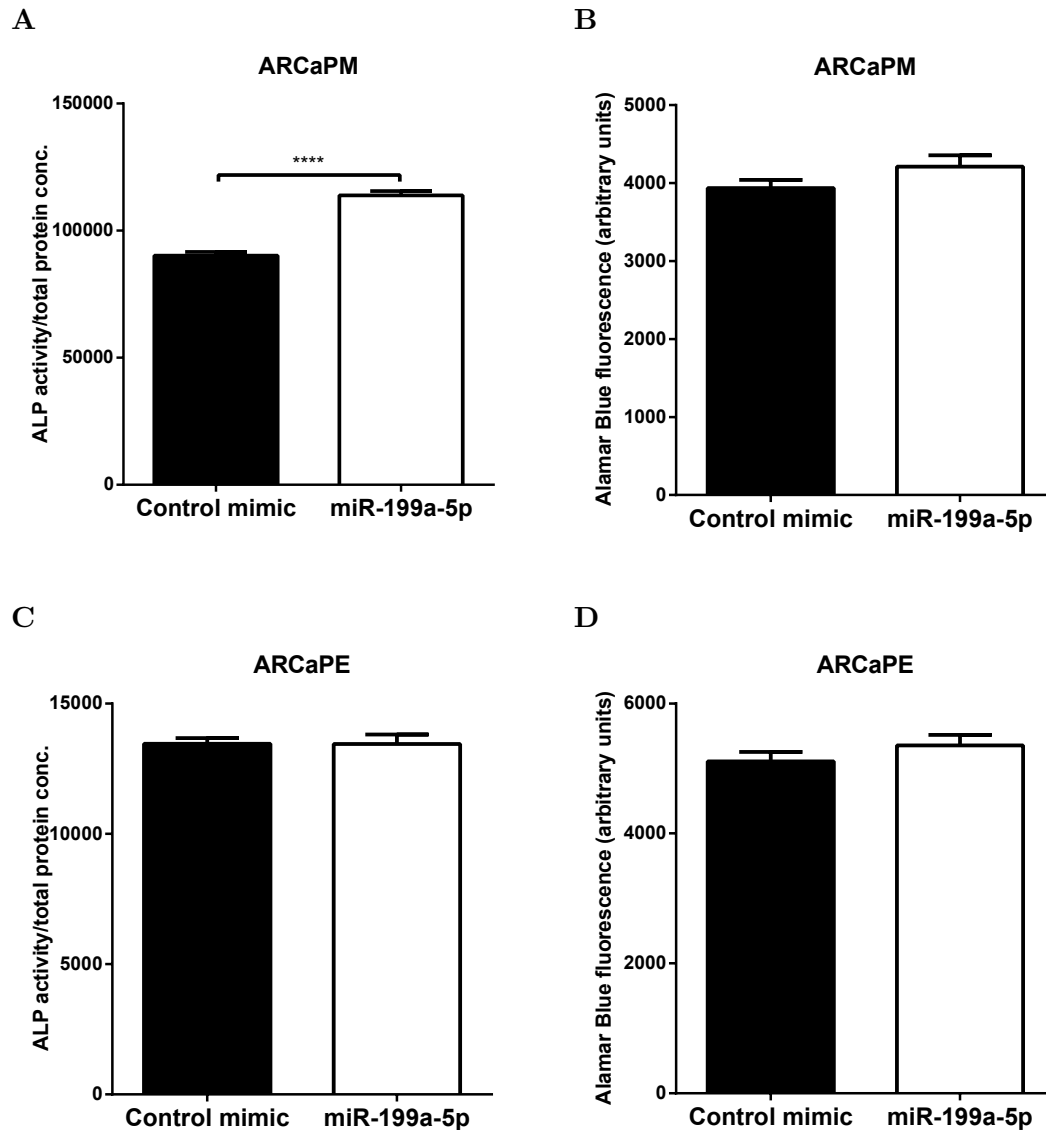


Figure 5.9: Transfection of miR-199a-5p induces increased ALP activity in ARCaPM cells, but has no effect on viability (A) MiR-199a-5p mimic transfection (50 nM) in ARCaPM cells and ALP activity fluorescence assay 72 hours following transfection. (B) Cell viability in miR-199a-5p or control mimic-transfected ARCaPM cells, using Alamar Blue assay. (C) MiR-199a-5p mimic transfection in ARCaPE cells and ALP activity fluorescence assay 72 hours following transfection. (D) Cell viability in miR-199a-5p or control mimic-transfected ARCaPE cells, using Alamar Blue assay. (Data are from an experiment in triplicates shown as mean $\pm$ S. E. ** P < 0.01, *** P < 0.001)

5.5 Summary

Stable expression of miR-203 was confirmed in ARCaPM cells by real-time RT-PCR. ALPL and RUNX2 mRNA expression were down-regulated in miR-203 over-expressing cells compared to control ARCaPM cells. This was accompanied by a marked decrease in mineralization and a significant reduction in alkaline phosphatase activity in miR-203 overexpressing ARCaPM cells compared to control cells. Small RNA sequencing identified 57 microRNAs that are up-regulated more than 2 fold in ARCaPE compared to ARCaPM cells. Conversely, 68 microRNAs were up-regulated more than 2 fold in ARCaPM compared to ARCaPE cells. A number of these microRNAs are known to be co-expressed as clusters; each cluster is present in the same genomic locus. Five microRNA clusters were found to be highly expressed in ARCaPE whereas 2 microRNA clusters were expressed at higher levels in ARCaPM cells. Real-time RT-PCR was used to confirm the expression of miR-373. Transfection of miR-373 mimic in ARCaPM cells resulted in a strong up-regulation of the microRNA. The mRNA expression of the epithelial marker E-cadherin was strongly upregulated in miR-373 overexpressing cells compared to control mimic transfected ARCaPM cells. In contrast, the expression of the mesenchymal markers vimentin and ZEB1 were significantly down-regulated in miR-373 overexpressing ARCaPM cells. An ALP activity-based screen was developed to identify microRNAs that regulate osteomimicry in ARCaPM cells. Using this screen on a pilot set of 80 microRNAs from a mimic library, two microRNAs, miR-199a-5p and miR-212, were identified as potential candidates. A validation experiment with miR-199a-5p demonstrated that it is indeed a positive regulator of ALP activity.

5.6 Conclusions

MicroRNAs are known to be important mediators of signalling pathways in a number of cancers. The microRNA miR-203 was shown to down-regulate bone metastasis in an osteolytic PC3 xenograft mouse model. MiR-203 is down-regulated in metastatic human prostate cancer samples emphasizing the clinical relevance of this microRNA. Our data demonstrated that miR-203 overexpression down-regulated the expression ALPL mRNA in ARCaPM cells. MiR-203 strongly down-regulated *in vitro* mineralization and ALP activity in ARCaPM cells. This suggests an important role for miR-203 in the regulation of osteoblastic bone disease in prostate cancer, in addition to metastasis. It was previously reported to directly target Survivin and Runx2 at their respective 3'-UTRs and repress their expression at a post-transcriptional level[163]. This is congruent with our data showing the down-regulation of Runx2 mRNA in ARCaPM cells. We aimed to identify a further set of microRNAs that are associated with EMT; due to their marked differences in metastatic ability ARCaPE and ARCaPM cells are a useful model to study this. Our RNA sequencing data revealed that a large number of microRNAs are differentially expressed between the two cell lines. Co-expression of a number of microRNAs in clusters strengthened our findings as it indicates a co-ordinated regulation of microRNA expression and suggests that they are not artifacts of RNA sequencing. MicroRNAs that were found to be highly expressed in ARCaPE, especially the miR-372 and miR-302 clusters are embryonic stem cell-specific, and are shown to induce mesenchymal-epithelial transition. This suggests that in addition to an association with epithelial phenotype, these two microRNA clusters play a causal role in the maintenance of the epithelial state. Further, many members of the miR-372 and miR-302 clusters share a common seed sequence. As the seed sequence of a microRNA is a crucial determinant of

its target specificity, this implies that the miR-302 and miR-373 clusters may be targeting a common set of mRNAs. Despite the seeming importance of these two clusters in the ARCaPE and ARCaPM model, few studies have demonstrated a change in these microRNAs in human prostate cancer metastatic samples. On the other hand, very few groups have studied differential expression of microRNAs in bone metastatic samples. All members of the miR-200 family, including miR-200a, -200b and -200c, are well established as epithelial markers and mediators of mesenchymal-epithelial transition. Intriguingly however, the expression of the miR-200b cluster, which includes miRs-200a, -200b and -429 was significantly higher in the mesenchymal ARCaPM compared to the epithelial ARCaPE cells. This suggests that (a) these microRNAs do not play a crucial role in regulating EMT in the ARCaPE/M model or (b) that the effect of other microRNAs or coding genes strongly eclipses their regulatory effect. It is important to note that 2 studies identifying circulatory microRNA biomarkers in prostate cancer show a higher expression of miR-200b in metastatic samples suggesting a more complex role for this microRNA. MiR-200c on the other hand, present at a different genomic locus, was highly expressed in ARCaPE cells. MiR-373 is expressed at a very low level in ARCaPM cells. Overexpression of this microRNA resulted in an increase in epithelial markers and down-regulation of mesenchymal markers indicating a regulatory role in EMT. MiR-373 has previously been shown to upregulate the expression of E-cadherin in PC3 cells by acting directly at its promoter[242]. Taken together, the results of this study demonstrate the important role of miR-373 as a strong mediator of MET and show that identification of differentially expressed microRNAs in the ARCaPE/M model will lead to the elucidation of functionally relevant microRNA regulatory mechanisms. However, its expression in subsets of human prostate cancer patients is not known and is therefore an important area of future studies. The development of a novel ALP activity-based microRNA mimic

library screen in prostate cancer cells is presented here. Given my results showing the important role of ALPL expression and ALP activity in prostate cancer cells (Chapter 3), an unbiased screening with a mimic library has the potential to identify novel signalling pathways mediated by microRNAs. Expression analysis using small RNA-seq, while useful in identifying EMT-associated microRNAs, does not ensure that the differentially expressed microRNAs are functionally important or that they play a causal or regulatory role in EMT. However, these data in combination with the ALP activity-based functional screen can identify such microRNAs that can induce or inhibit EMT. High-throughput assays are not a simple scaling up of low-throughput assays. They require thorough optimisation to eliminate spurious effects due to a multitude of unexpected factors like excessive evaporation of growth medium and consequent cell death at the edges of a 96-well plate, pipetting errors at various stages and plate-to-plate variation. In addition, analysis of such high-throughput data needs to account for multiple comparisons to generate robust data that can produce a high success rate in subsequent validation studies. In the screen described here, two microRNAs, miR-199a-5p and miR-212, were identified as regulators of ALP activity and this was confirmed in the case of the former in a subsequent validation study. However, it has to be noted that the upregulation of ALP activity by miR-199a-5p mimic transfection is modest and only a complete analysis of the entire mimic library may reveal stronger and more functionally important candidates. Further work is being done in this direction to optimise this assay to generate more reliable data.

5.6.1 Limitations and future studies

Unlike siRNAs/shRNAs that are designed to specifically target a particular mRNA, each microRNA may target many mRNAs; hence it is possible that candidates identified from the ALP activity-based functional screen may robustly regulate

ALP expression and activity but at the same time, have no effect on EMT or cell survival. However, a combination of the microRNA expression data (RNA-seq) and functional screening data may give more meaningful data. Future work involves further optimisation (for example, reverse transfection), to ensure reproducibility, and completion of the mimic library functional screen.

*The combination of some data and an aching desire
for an answer does not ensure that a reasonable
answer can be extracted from a given body of data.*

—John Tukey, *Sunset Salvo*

6

Conclusions and significance

Epithelial-mesenchymal transition is an important process that drives prostate cancer progression and is thought to play a crucial role in the spread of tumour cells to distant sites like bone. This study explores three mechanisms of regulation of EMT, interlinked by common themes (Appendix C).

Osteomimicry has long been thought to have an important role in prostate cancer progression and bone metastasis[171][172]. However, while alkaline phosphatase is considered an important marker of metastatic bone disease due to its role in bone turnover, its role in cancer progression is virtually unknown. It has been previously shown that increased expression of alkaline phosphatase is seen in germ cell tumours[243] but these studies did not show evidence for a causal role, nor did they delve into the mechanistic details. Hence, this is the first study to demonstrate that ALPL plays an important role in the maintenance of epithelial-mesenchymal transition and cell survival in prostate cancer cells. Further, these data suggest that ALPL activity may be regulated to a modest degree by Snail

in prostate cancer cells. It is well known in the bone research community that the transcription of ALPL is regulated by Runx2. To my knowledge, this is the first study to identify the regulation of ALPL expression by another transcription factor. Runx2 expression has been shown to be associated with prostate cancer progression and metastasis. As ALPL is a direct transcriptional target of Runx2, it is reasonable to postulate that the pro-metastatic effects of Runx2 may be at least partially mediated by an upregulation of ALPL expression and activity. However, there is very little literature that explores the functional role of ALPL in prostate cancer; the majority of reports that mention ALPL explore its role as a marker of new bone formation. A recent report demonstrates that a loss of epithelial character is associated with increased alkaline phosphatase expression in circulating prostate cancer cells from human patients[244]. Considering the crucial role of ALPL in EMT as shown in this thesis, further exploration of its expression in normal and cancerous prostate tissues is warranted. In addition, this raises interesting questions about the role of ALPL in normal physiology; recently, it has been shown that shRNA-mediated knockdown of ALPL in MC3T3-E1 mouse osteoblast cells results in decreased viability, increased adhesion to culture surface *in vitro* and increased apoptosis[245]. Increased apoptosis was also observed in the thymus of ALPL knockout mice[246]. These, in conjunction with the findings in this thesis, suggests that alkaline phosphatase may play a more fundamental role in the survival of multiple cell types in addition to its enzymatic function in bone mineralization.

Tissue non-specific alkaline phosphatase is routinely used as a test for pluripotency in embryonic stem cells and embryonic carcinoma cells[247][248]. High ALP expression in ovarian carcinoma cells was associated with cancer stemness and multilineage differentiation potential. This cancer stemness was reduced when alkaline phosphatase activity was blocked with levamisole, an inhibitor of ALP[249]. This

suggests that alkaline phosphatase may have a role to play in cancer stemness in prostate cells too, in which case, inhibition of alkaline phosphatase activity could be a viable strategy to target tumour cells.

All the data presented in this thesis describes the knockdown and inhibition of tumour-derived ALP. The role of host-derived ALP, or in cultured cells *in vitro*, the role of ALP in the growth medium is not clear. Levamisole treatment of AR-CaPM cells results in cell death. However, preliminary data of ARCaPE cells treated with or without porcine ALP in the growth medium (Fig. A.5) suggests that extracellular ALP has no effect on the viability of those cells. This could be because of a number of reasons: 1) ARCaPE cells may not always be able to revert to a mesenchymal state. 2) Extracellular ALP, may not have any effect on cell survival. However, as levamisole mainly inhibits extracellular alkaline phosphatase, it can only be assumed that the ALP-inhibitory effect of levamisole would be limited to extracellular and plasma membrane-bound ALP[250]. 3) Porcine ALP may not have exactly the same effects as human ALP on human prostate cancer cells. Hence, this experiment needs to be repeated using human recombinant ALP. In addition, a more pertinent experiment would be to treat ALPL-knockdown AR-CaPM cells with human recombinant ALP, to determine if the latter can “rescue” prostate cancer cells from the loss of ALPL expression. If it can be confirmed in these ways that extracellular ALP can substitute for the loss of tumour-derived ALPL expression, the contribution of host-derived ALP to prostate cancer metastasis could be an very interesting question to be answered. Considering the fact that the most common sites of prostate cancer metastasis, which are bone, liver, lungs and adrenal glands, have high expression levels of ALPL (Protein Atlas[251]), it may be speculated that tumour cells tend to colonize these specific organs due to high ALP activity in the microenvoronment, inducing EMT and cell proliferation. This again could mean that inhibition of ALP activity would be an effective anti-

tumour strategy in metastatic sites. Recently, it was reported that alkaline phosphatase replacement can be performed successfully in human hypophosphatasia patients, suggesting that targeting this enzyme can be a viable option in prostate cancer[252].

Levamisole is known to affect multiple signalling pathways and hence it is possible that its effects may not be specific to ALP inhibition. If this is the case, an alternative mechanism may be postulated speculating on a non-enzymatic role of ALPL in the regulation of cell signalling. It is interesting to note that ALPL as well as a number of EMT markers including Snail, Slug, Transforming Growth Factor- β Receptor 2 and Runx2 are all validated targets of the microRNA miR-204[253][254][255][256][257]. This raises the possibility of a microRNA-mediated regulation of growth and migration-promoting gene expression by ALPL. Pandolfi et al. recently proposed a novel regulatory mechanism mediated by microRNAs, which they have termed as the competing endogenous RNA (ceRNA) hypothesis, according to which, mRNAs that are targeted by the same set of microRNAs are co-expressed. They demonstrated that PTEN and the corresponding pseudogene PTENP1 are targeted by the same set of microRNAs and that PTEN and PTENP1 are co-expressed in various cancers[258]. It is possible that in the same manner, ALPL may regulate one or more mesenchymal markers through shared common sequences in their 3' UTRs, allowing them to be targeted by the same set of microRNAs. The co-expression of Snail and ALPL supports this possibility, due to their sharing of validated miR-204 target sites. Hence, a rescue of epithelial phenotype in ARCaPM cells by overexpression of ALPL 3' or 5' UTR, but not by overexpression of the ALPL coding region, would be preliminary evidence to verify this hypothesis and future work will need to address this. It is interesting to note that considering the remarkable effects of ALPL knockdown on prostate cancer cell and osteoblast survival *in vitro*, the development of TNAP knockout mice is

largely unimpaired except for defects in bone mineralization and seizures. While this may be explained by the possibility that the role of ALPL by other enzymes redundantly *in vivo*, it may instead be possible that knockout and knockdown of the gene produce two distinct phenotypes. TNAP knockout studies in mice were performed using a strategy that produces a truncated coding region, leaving the 5' UTR intact[259][49][246], whereas ALPL knockdown resulted in degradation of the entire mRNA, allowing speculation that ALPL may play a microRNA-mediated coding-independent role as described above.

Although purinergic signalling in this study was explored in the context of its role as a potential mediator of ALPL-regulated EMT, there is justification for its study from the perspective of the effect of the tumour microenvironment on tumour cells; purinergic receptors have a role in prostate cancer cell survival and are therefore of interest independent of ALPL. PC3 cells express a number of P2X and P2Y receptors and ATP has effects on both viability and cell survival[228][260]. Further, an ATP dosing regimen in a mouse model of prostate cancer inhibited tumour growth[229]. Hence, the finding that extracellular ATP induces MET in ARCaPM cells suggests novel mechanisms for the regulation of prostate cancer metastasis by purinergic signalling. Enzymes that dephosphorylate extracellular nucleotides are called ecto-nucleotidases; 5'-ectonucleotidase (CD73) is overexpressed in both prostate cancer samples[261] as well as the tumour-associated stroma[262] and the ecto-nucleotidase CD39/ENTPD1 has been shown to promote tumour cell growth by eliminating extracellular ATP[263]. Interestingly, prostatic acid phosphatase has been found to be expressed by neuronal cells and it mediates pain signalling through its ecto-nucleotidase activity[264][265]. These reports suggest the possibility that tumour cells may be escaping the growth- and metastasis-inhibitory effects of extracellular ATP and further studies on these and other ecto-nucleotidases are warranted.

The microRNA studies presented here have identified novel mechanisms regulating EMT and alkaline phosphatase expression. This is significant in the context of the emerging importance of microRNAs as biomarkers, both in serum and tissue samples. Further, as proposals accumulate advocating the use of microRNAs as therapeutic targets, there is an imperative to understand all the biological functions of each microRNA in detail to avoid unintended consequences. This is of greater concern in the case of microRNAs, in contrast to other protein-coding genes, due to their ability to regulate multiple genes. This study demonstrates proof-of-concept for the use of high-throughput functional screening techniques to delineate novel signalling mechanisms. The combination of high-throughput quantitation of cell shape and screening for alkaline phosphatase provide powerful tools to this end. Previous reports have shown that high-throughput screens for ALP activity is an effective strategy to identify microRNAs regulating mesenchymal stem cell differentiation to the osteoblast lineage[266]. Hence, it is hoped that similar techniques applied in the context of ALP-regulated EMT would pay high dividends. Future work in this direction will involve performing ALP activity screening for a complete mimic library of known microRNAs and identifying novel microRNA regulators of ALP activity and/or EMT. Candidates thus identified, miR-199a-5p for example, can be further investigated to determine their expression and role in prostate cancer progression and bone metastasis. In addition, microRNAs are known to regulate the expression of P2 purinergic receptors in cancer cells[267][268] and it would be interesting to explore the role of microRNA candidates (from the mimic library screen) in the regulation of purinergic signalling.

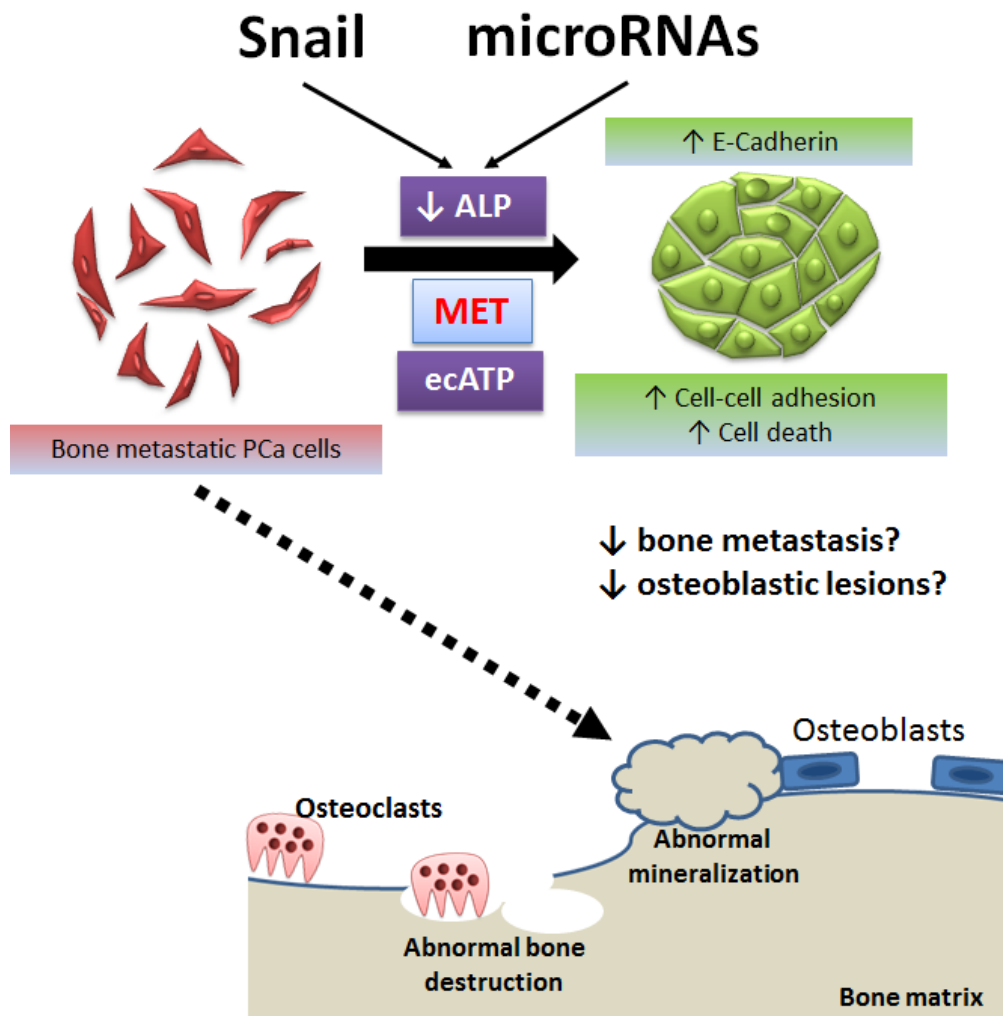


Figure 6.1: Summary The main findings in this thesis are (1) the identification of a novel role for ALPL in regulating EMT, (2) the regulation of ALPL by Snail and microRNAs, and (3) the induction of MET by extracellular ATP (ecATP), in prostate cancer. Ongoing and future studies will address the significance of ALPL-regulated EMT in prostate cancer bone metastasis *in vivo*.

This thesis describes the identification of a novel role for tumour-derived ALPL in the regulation of EMT in prostate cancer cells (Chapter 3). The possibility of ATP acting as a mediator of ALPL's role was explored (chapter 4). Finally, the role of microRNAs in the regulation of EMT and ALP activity was studied (chapter 5). Taken together (Fig. 6.1), these data represent novel findings that expand the boundaries of the prostate cancer bone metastasis field, but also raise some intriguing questions which would be the focus of future studies. In addition, a high-throughput migration assay is in development in collaboration with the High-throughput Screening Group (headed by Dr. Daniel Ebner, Target Discovery Institute, University of Oxford). This in combination with the high-throughput screening approaches described in this thesis are part of a larger comprehensive project (Fig. 6.2) that aims to identify novel microRNA regulators of EMT, migration and ultimately, bone metastasis in prostate cancer.

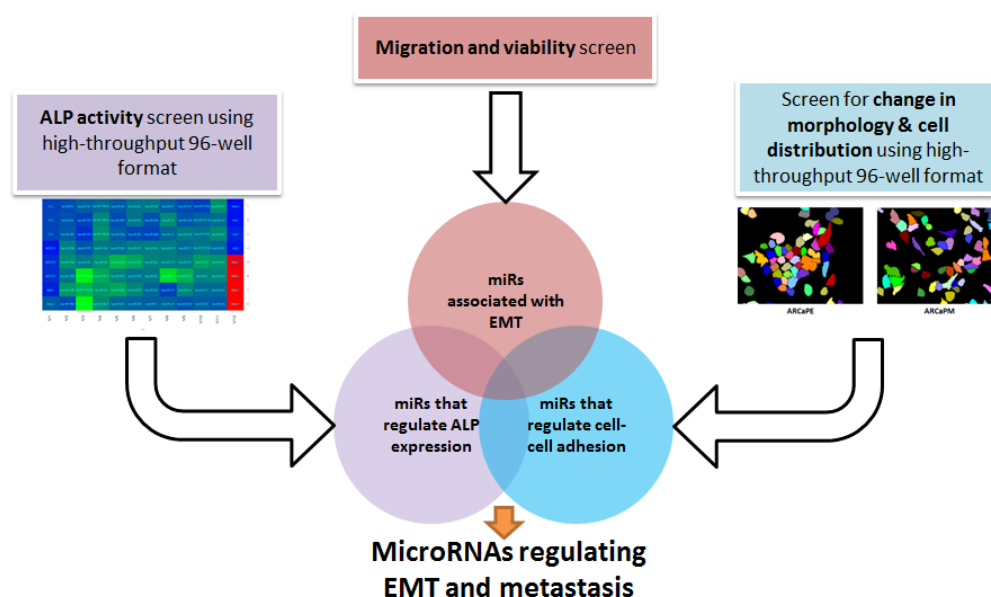


Figure 6.2: Combination of high-throughput screening techniques to identify microRNA regulators of EMT and migration in prostate cancer.

A

Appendix

A.1 Levamisole treatment decreased 22Rv1 cell viability

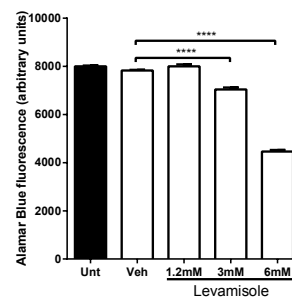


Figure A.1: Levamisole induced a reduction in cell viability in 22Rv1 prostate cancer cells Cell viability in levamisole-treated ARCaPM cells was measured using Alamar Blue assay. (Data are shown as mean $\pm$ S. E. **** P <0.0001)

A.2 ALPL expression is weakly negatively correlated with E-cadherin and ZEB1 expression in human prostate cancer samples

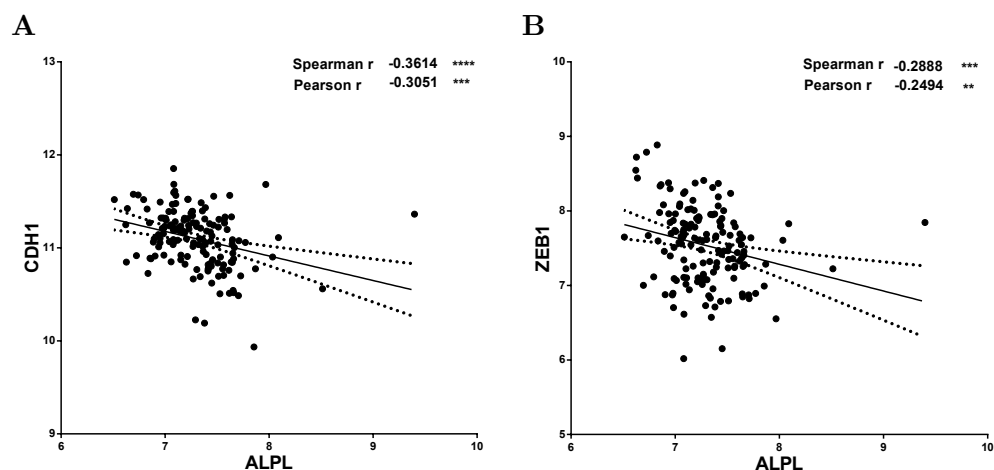


Figure A.2: Correlation of ALPL with CDH1 and ZEB1 gene expression in human prostate cancer samples (A) mRNA expression data was mined from a previously published dataset. ALPL mRNA expression was plotted against CDH1 expression or (B) ZEB1 expression. Solid lines represent linear regression and the dotted lines are 95% confidence intervals. (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)

A.3 Apyrase degrades ATP *in vitro*

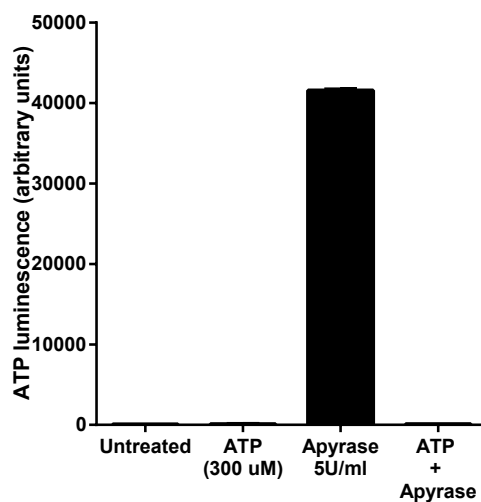


Figure A.3: Apyrase degrades ATP in serum-free medium Serum-free medium containing ATP (300 μ M) or control medium, was incubated for 2 hours with or without apyrase (5 U/ml) and ATP was measured using a luciferin-luciferase based bioluminescence assay kit.

A.4 Apyrase does not rescue ARCaPM cells from levamisole-induced decrease in viability

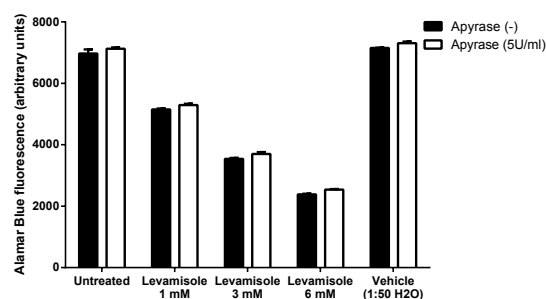


Figure A.4: Exogenous apyrase has no effect on levamisole-induced reduction in cell viability in ARCaPM cells Cell viability was measured in ARCaPM cells grown in serum-free medium, treated with levamisole alone or in combination with apyrase for 24 hours, using Alamar Blue assay.

A.5 Exogenously added porcine ALP has ectonucleotidase function *in vitro*, but has no effect on ARCaPE cell viability

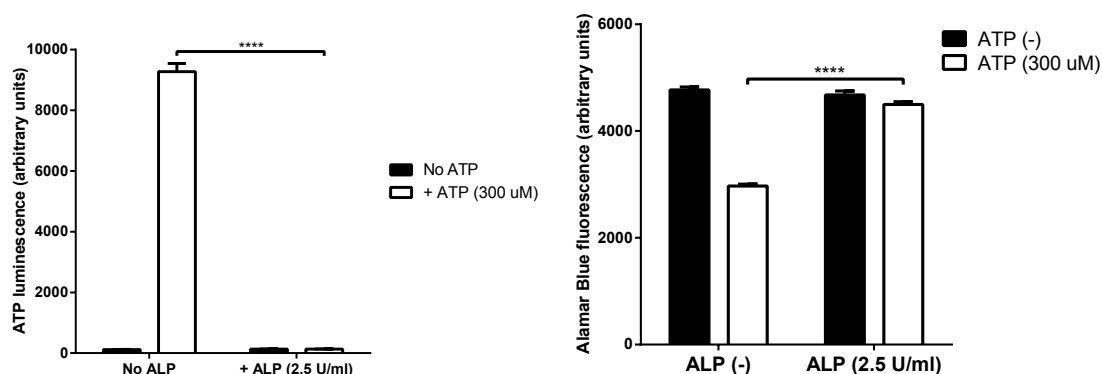


Figure A.5: Exogenous porcine ALP has no effect on ARCaPE cell viability (A) ATP was measured using a luciferin-luciferase based bioluminescence assay in serum-free medium containing ATP or control medium with or without porcine ALP. (B) Cell viability was measured in ARCaPE cells treated with ATP alone or in combination with porcine ALP, using Alamar Blue assay. (Data are shown as mean $\pm$ S. E. *** P < 0.001)

A.6 Optimisation of mimic library screen

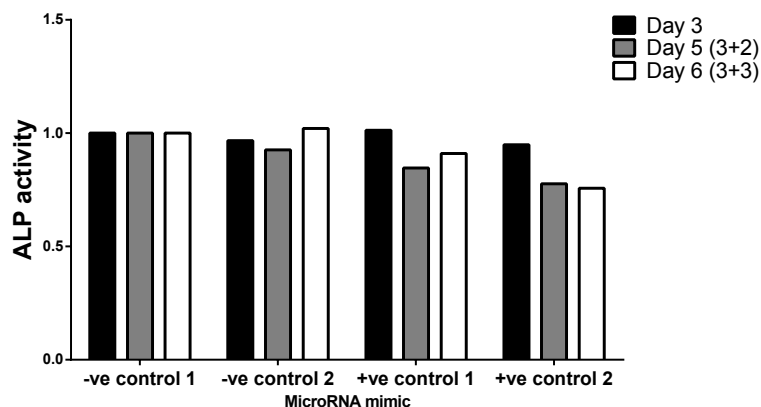


Figure A.6: An example of the optimisation performed for the development of the microRNA mimic library screen in 96-well format. 50 nM each of negative or positive microRNA mimic controls were transfected into ARCaPM cells as described. Conditioned medium was collected from the transfected cells at 3, 5 or 6 days following transfection. When collecting at 5 or 6 days, culture medium was changed on the third day to ensure that the cells remain healthy. From these optimisation experiments, a duration of 6 days following mimic transfection was found to produce the most significant changes in ALP activity. (Data are shown as mean of 5 replicates.)

B

Appendix

B.1 R code for data mining

```
install.packages('cgdsr')
library(cgdsr)
# Create CGDS object
mycgds = CGDS("http://www.cbioportal.org/public-portal/")

# Get available case lists (collection of samples) for a given
  cancer study
mycancerstudy = getCancerStudies(mycgds)[54,1]
mycaselist = getCaseLists(mycgds,mycancerstudy)[23,1]

# Get available genetic profiles
mygeneticprofile = getGeneticProfiles(mycgds,mycancerstudy)[4,1]

# Get data slices for a specified list of genes, genetic profile
  and case list
my.ZEB1expMSKCC <- getProfileData(mycgds,c('ZEB1'),mygeneticprofile
  ,mycaselist)
my.ALPLexpMSKCC <- getProfileData(mycgds,c('ALPL'),mygeneticprofile
  ,mycaselist)
```

```

my.SNAI1expMSKCC <- getProfileData(mycgds,c('SNAI1'),
  mygeneticprofile,mycaselist)
my.CDH1expMSKCC <- getProfileData(mycgds,c('CDH1'),mygeneticprofile
  ,mycaselist)
my.PTENexpMSKCC <- getProfileData(mycgds,c('PTEN'),mygeneticprofile
  ,mycaselist)

# Get clinical data for the case list
myclinicaldata = getClinicalData(mycgds,mycaselist)

my.ALPLinPROSTATE <- my.ALPLexp[grep("PROSTATE", rownames(my.
  ALPLexp)), ]
my.ALPLinPROSTATE <- as.matrix(my.ALPLinPROSTATE, ncol=1)

row.names(my.ALPLinPROSTATE) <- rownames(my.ALPLexp)[grep("PROSTATE
  ", rownames(my.ALPLexp))]

```

B.2 Pyridoxine concentration in growth media

Table B.1: Pyridoxine concentration in growth media

Growth medium	Pyridoxine.HCl (mg/l)
RPMI 1640 (Sigma, Cat. R0883)	1
DMEM (Sigma, Cat. 6046)	4.04
T-medium (Life Technologies, Cat. 02-0056)	3.212

C

Appendix

Awards and funding

Best Poster Award, Nuffield Dept. of Surgical Sciences, University of Oxford, Departmental Away Day, September 2014 (Selected based on competitive review)

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New Investigator Award, Bone Research Society, UK, Annual meeting, June 2014 (Selected based on abstract ranking)

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Rao, S. R., Snaith, A. E., Yapp, C., Orriss, I., Hamdy, F. C. and Edwards, C. M. Extracellular ATP induces Mesenchymal-Epithelial Transition (MET) in prostate cancer cells. *Annual Away Day of the Nuffield Department of Surgical Sciences* (Sept. 2014).

Rao, S. R., Kratschmer, P., Edwards, J. R., Hamdy, F. C. and Edwards, C. M. MiR-373 inhibits functional osteomimicry in osteoblastic prostate cancer cells. *Annual Meeting of the Bone Research Society* (June 2014).

Rao, S. R., Lwin, S. T., Kratschmer, P., Yapp, C., Edwards, J. R., Hamdy, F. C. and Edwards, C. M. MicroRNAs regulate functional osteomimicry, promoting prostate cancer-induced bone disease. *29th Annual Congress of the European Association of Urology* (Apr. 2014).

Rao, S. R., Kratschmer, P., Yapp, C., Edwards, J. R., Hamdy, F. C. and Edwards, C. M. The role of microRNAs in functional osteomimicry in prostate cancer cells. *Bone Research Society/British Orthopaedic Research Society Joint Meeting* (Sept. 2013).

Rao, S. R., Hamdy, F. C. and Edwards, C. M. The role of microRNAs in functional osteomimicry in prostate cancer cells. *BSMB Spring Meeting, Epigenetics in Matrix Biology and Disease* (Mar. 2013).

Bibliography

1. Rothfuss, P. *The Name Of The Wind: The Kingkiller Chonicle: Book 1* 151 (Hachette Littlehampton. Kindle Edition., Apr. 18, 2010) (cit. on p. [F](#)).
2. Coleman, R. E. Skeletal complications of malignancy. *Cancer* **80**, 1588–1594 (Oct. 1997) (cit. on p. [1](#)).
3. Palmer, R. Nicolò Massa, his family and his fortune. *Medical History* **25**, 385–410 (Oct. 1981) (cit. on p. [2](#)).
4. Vesalius, A. *De Humani Corporis Fabrica Libri Septem* (Basileae : ex officina Ioannis Oporini, 1543) (cit. on pp. [2](#), [3](#)).
5. Schultz, M., Parzinger, H., Posdnjakov, D. V., Chikisheva, T. A. & Schmidt-Schultz, T. H. Oldest known case of metastasizing prostate carcinoma diagnosed in the skeleton of a 2,700-year-old Scythian king from Arzhan (Siberia, Russia). *International Journal of Cancer* **121**, 2591–2595 (Dec. 2007) (cit. on p. [2](#)).
6. Prates, C., Sousa, S., Oliveira, C. & Ikram, S. Prostate metastatic bone cancer in an Egyptian Ptolemaic mummy, a proposed radiological diagnosis. *International Journal of Paleopathology* **1**, 98–103 (Oct. 2011) (cit. on p. [2](#)).
7. Young, H. H. The early diagnosis and radical cure of carcinoma of the prostate. Being a study of 40 cases and presentation of a radical operation which was carried out in four cases. *The Journal of Urology* **168**, 914–921 (Sept. 1905) (cit. on p. [2](#)).
8. Huggins, C. & Hodges, C. V. Studies on Prostatic Cancer. I. The Effect of Castration, of Estrogen and of Androgen Injection on Serum Phosphatases in Metastatic Carcinoma of the Prostate. *Cancer Research* **1**, 293–297 (Apr. 1941) (cit. on p. [2](#)).
9. Denmeade, S. R. & Isaacs, J. T. A history of prostate cancer treatment. *Nature Reviews Cancer* **2**, 389–396 (May 2002) (cit. on p. [2](#)).

10. Cancer Research UK. *Cancer incidence for common cancers* Document. The latest incidence statistics for the most common cancers (excluding non-melanoma skin cancer) diagnosed in the UK. Sept. 2014 (cit. on pp. [4](#), [5](#)).
11. Cancer Research UK. *Cancer mortality for common cancers* Document. The latest UK cancer mortality statistics for the most common cancers from the Statistics Team at Cancer Research UK. Sept. 2014 (cit. on pp. [4](#), [5](#)).
12. Cancer Research UK. *Cancer statistics: Key facts (2014)* (cit. on p. [4](#)).
13. Nørgaard, M., Jensen, A. Ø., Jacobsen, J. B., Cetin, K., Fryzek, J. P. & Sørensen, H. T. Skeletal Related Events, Bone Metastasis and Survival of Prostate Cancer: A Population Based Cohort Study in Denmark (1999 to 2007). *The Journal of Urology* **184**, 162–167 (July 2010) (cit. on p. [4](#)).
14. Remzi, M., Waldert, M. & Djavan, B. Prostate cancer in the ageing male. *The Journal of Men's Health & Gender* **1**, 47–54 (May 2004) (cit. on p. [4](#)).
15. Luengo-Fernandez, R., Leal, J., Gray, A. & Sullivan, R. Economic burden of cancer across the European Union: a population-based cost analysis. *The Lancet* **14**, 1165–1174 (Nov. 2013) (cit. on p. [4](#)).
16. Kheirandish, P. & Chinegwundoh, F. Ethnic differences in prostate cancer. *British Journal of Cancer* **105**, 481–485 (Aug. 2011) (cit. on p. [4](#)).
17. Mottet, N., Bastian, P., Bellmunt, J., van den Bergh, R., Bolla, van Casteren, N., Cornford, P., Joniau, S., Mason, M., Matveev, V., van der Kwast, T., van der Poel, H., Rouvière, O. & Wiegel, T. Guidelines on Prostate Cancer. *European Association of Urology* (2014) (cit. on pp. [4](#), [8](#), [10](#), [11](#)).
18. True, L. & Vakar-Lopez, F. *Recent advances in prostate cancer* (eds Tindall, D. J. & Scardino, P. T.) chap. Pathology of prostate (World Scientific Publishing, 2011) (cit. on pp. [6](#), [37](#)).
19. Owen, D. H. & Katz, D. F. A Review of the Physical and Chemical Properties of Human Semen and the Formulation of a Semen Simulant. *Journal of Andrology* **26**, 459–469 (July 2005) (cit. on p. [6](#)).
20. Gevaert, T., Lerut, E., Joniau, S., Franken, J., Roskams, T. & De Ridder, D. Characterization of subepithelial interstitial cells in normal and pathological human prostate. *Histopathology* **65**, 418–428 (Sept. 2014) (cit. on p. [6](#)).
21. Lu, T.-L., Huang, Y.-F., You, L.-R., Chao, N.-C., Su, F.-Y., Chang, J.-L. & Chen, C.-M. Conditionally Ablated Pten in Prostate Basal Cells Promotes Basal-to-Luminal Differentiation and Causes Invasive Prostate Cancer in

- Mice. *The American Journal of Pathology* **182**, 975–991 (Mar. 2013) (cit. on p. 6).
22. McNeal, J. E. The zonal anatomy of the prostate. *The Prostate* **2**, 35–49 (Jan. 1981) (cit. on p. 6).
23. Amin, M., Khalid, A., Tazeen, N. & Yasoob, M. Zonal Anatomy of Prostate. *Annals of King Edward Medical University* **16** (Apr. 2011) (cit. on p. 6).
24. Garber, K. A Tale of Two Cells: Discovering the Origin of Prostate Cancer. *Journal of the National Cancer Institute* **102**, 1528–1535 (Oct. 2010) (cit. on p. 7).
25. Taylor, R. A., Toivanen, R., Frydenberg, M., Pedersen, J., Harewood, L., Australian Prostate Cancer Bioresource, Collins, A. T., Maitland, N. J. & Risbridger, G. P. Human epithelial basal cells are cells of origin of prostate cancer, independent of CD133 status. *Stem Cells* **30**, 1087–1096 (June 2012) (cit. on p. 7).
26. Wang, Z. A., Toivanen, R., Bergren, S. K., Chambon, P. & Shen, M. M. Luminal Cells Are Favored as the Cell of Origin for Prostate Cancer. *Cell Reports* **8**, 1339–1346 (Nov. 2014) (cit. on p. 7).
27. Yin, J. J., Pollock, C. B. & Kelly, K. Mechanisms of cancer metastasis to the bone. *Cell Research* **15**, 57–62 (Jan. 2005) (cit. on p. 7).
28. Kuriyama, M., Wang, M. C., Papsidero, L. D., Killian, C. S., Shimano, T., Valenzuela, L., Nishiura, T., Murphy, G. P. & Chu, T. M. Quantitation of prostate-specific antigen in serum by a sensitive enzyme immunoassay. *Cancer Research* **40**, 4658–4662 (Dec. 1980) (cit. on p. 8).
29. Barry, M. J. Screening for Prostate Cancer — The Controversy That Refuses to Die. *New England Journal of Medicine* **360**, 1351–1354 (Mar. 2009) (cit. on p. 8).
30. Yang, S., Liang, J., Lin, F., Kao, C., Lin, C. & Lee, C. Comparing whole body 18F-2-deoxyglucose positron emission tomography and technetium-99m methylene diphosphonate bone scan to detect bone metastases in patients with breast cancer. *Journal of Cancer Research and Clinical Oncology* **128**, 325–328 (June 2002) (cit. on p. 9).
31. Liao, W.-H., Lin, S.-H. & Wu, T.-T. A 70-year-old male having advanced prostate cancer presenting with hypercalcemia and diffuse osteoblastic bone metastases: a case report. *Cases Journal* **2**, 54 (Jan. 2009) (cit. on p. 9).

32. Humphrey, P. A. Gleason grading and prognostic factors in carcinoma of the prostate. *Modern Pathology* **17**, 292–306 (Feb. 2004) (cit. on p. [10](#)).
33. Heidenreich, A., Bastian, P. J., Bellmunt, J., Bolla, M., Joniau, S., van der Kwast, T., Mason, M., Matveev, V., Wiegel, T., Zattoni, F. & Mottet, N. EAU Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent—Update 2013. *European Urology* **65**, 124–137 (Jan. 2014) (cit. on p. [12](#)).
34. Heidenreich, A., Bastian, P. J., Bellmunt, J., Bolla, M., Joniau, S., van der Kwast, T., Mason, M., Matveev, V., Wiegel, T., Zattoni, F. & Mottet, N. EAU Guidelines on Prostate Cancer. Part II: Treatment of Advanced, Relapsing, and Castration-Resistant Prostate Cancer. *European Urology* **65**, 467–479 (Feb. 2014) (cit. on p. [12](#)).
35. Hanahan, D. & Weinberg, R. Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646–674 (Mar. 2011) (cit. on p. [13](#)).
36. Valastyan, S. & Weinberg, R. A. Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147**, 275–292 (Oct. 2011) (cit. on p. [13](#)).
37. Paget, S. The distribution of secondary growths in cancer of the breast. *The Lancet. Volume 1, Issue 3421* **133**, 571–573 (Mar. 1889) (cit. on p. [14](#)).
38. Fidler, I. J. The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited. *Nature Reviews Cancer* **3**, 453–458 (June 2003) (cit. on p. [14](#)).
39. Raggatt, L. J. & Partridge, N. C. Cellular and Molecular Mechanisms of Bone Remodeling. *Journal of Biological Chemistry* **285**, 25103–25108 (Aug. 2010) (cit. on p. [14](#)).
40. Sims, N. A. & Gooi, J. H. Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. *Seminars in Cell & Developmental Biology* **19**, 444–451 (Oct. 2008) (cit. on p. [15](#)).
41. Mundy, G. R. Metastasis: Metastasis to bone: causes, consequences and therapeutic opportunities. *Nature Reviews Cancer* **2**, 584–593 (Aug. 2002) (cit. on pp. [15](#), [19](#)).
42. Bonewald, L. F. Mechanosensation and Transduction in Osteocytes. *BoneKEY osteovision* **3**, 7–15 (Oct. 2006) (cit. on p. [15](#)).
43. Minguell, J. J., Erices, A. & Conget, P. Mesenchymal Stem Cells. *Experimental Biology and Medicine* **226**, 507–520 (June 2001) (cit. on p. [15](#)).

44. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y.-H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. & Kishimoto, T. Targeted Disruption of *Cbfa1* Results in a Complete Lack of Bone Formation owing to Maturation Arrest of Osteoblasts. *Cell* **89**, 755–764 (May 1997) (cit. on p. 15).
45. Conget, P. A. & Minguell, J. J. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *Journal of Cellular Physiology* **181**, 67–73 (Oct. 1999) (cit. on p. 15).
46. Ducy, P. *Cbfa1*: a molecular switch in osteoblast biology. *Developmental Dynamics* **219**, 461–471 (Dec. 2000) (cit. on p. 15).
47. Hecht, J., Seitz, V., Urban, M., Wagner, F., Robinson, P. N., Stiege, A., Dieterich, C., Kornak, U., Wilkening, U., Brieske, N., Zwingman, C., Kidess, A., Stricker, S. & Mundlos, S. Detection of novel skeletogenesis target genes by comprehensive analysis of a *Runx2*^{-/-} mouse model. *Gene Expression Patterns* **7**, 102–112 (Jan. 2007) (cit. on p. 15).
48. Asmann, Y. W., Necela, B. M., Kalari, K. R., Hossain, A., Baker, T. R., Carr, J. M., Davis, C., Getz, J. E., Hostetter, G., Li, X., McLaughlin, S. A., Radisky, D. C., Schroth, G. P., Cunliffe, H. E., Perez, E. A. & Thompson, E. A. Detection of redundant fusion transcripts as biomarkers or disease-specific therapeutic targets in breast cancer. *Cancer Research* **72**, 1921–1928 (Apr. 2012) (cit. on p. 16).
49. Waymire, K. G., Mahuren, J. D., Jaje, J. M., Guilarte, T. R., Coburn, S. P. & MacGregor, G. R. Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nature Genetics* **11**, 45–51 (Sept. 1995) (cit. on pp. 16, 80, 132).
50. Fedde, K. N., Blair, L., Silverstein, J., Coburn, S. P., Ryan, L. M., Weinstein, R. S., Waymire, K., Narisawa, S., Millán, J. L., MacGregor, G. R. & Whyte, M. P. Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *Journal of Bone and Mineral Research* **14**, 2015–2026 (Dec. 1999) (cit. on p. 16).
51. Anderson, H. C., Sipe, J. B., Hessle, L., Dharmyramaju, R., Atti, E., Camacho, N. P. & Millán, J. L. Impaired Calcification Around Matrix Vesicles of Growth Plate and Bone in Alkaline Phosphatase-Deficient Mice. *The American Journal of Pathology* **164**, 841–847 (Mar. 2004) (cit. on p. 16).
52. Narisawa, S., Wennberg, C. & Luis Millán, J. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but

- not the impaired bone mineralization. *The Journal of Pathology* **193**, 125–133 (Jan. 2001) (cit. on p. 17).
53. Wennberg, C., Hessle, L., Lundberg, P., Mauro, S., Narisawa, S., Lerner, U. H. & Millán, J. L. Functional Characterization of Osteoblasts and Osteoclasts from Alkaline Phosphatase Knockout Mice. *Journal of Bone and Mineral Research* **15**, 1879–1888 (Oct. 2000) (cit. on pp. 17, 54).
54. Mornet, E. Hypophosphatasia: the mutations in the tissue-nonspecific alkaline phosphatase gene. *Human Mutation* **15**, 309–315 (2000) (cit. on p. 17).
55. Harris, H. The human alkaline phosphatases: What we know and what we don't know. *Clinica Chimica Acta* **186**, 133–150 (Jan. 1990) (cit. on p. 17).
56. Linder, C. H., Narisawa, S., Millan, J. L. & Magnusson, P. Glycosylation differences contribute to distinct catalytic properties among bone alkaline phosphatase isoforms. *Bone* **45**, 987–993 (Nov. 2009) (cit. on p. 17).
57. Young, M. F. Bone matrix proteins: their function, regulation, and relationship to osteoporosis. *Osteoporosis international* **14 Suppl 3**, S35–42 (2003) (cit. on p. 17).
58. Boyce, B. F. & Xing, L. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Research & Therapy* **9 Suppl 1**, S1 (2007) (cit. on p. 18).
59. Bar-Shavit, Z. The osteoclast: A multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. *Journal of Cellular Biochemistry* **102**, 1130–1139 (Dec. 2007) (cit. on p. 18).
60. Blair, H. C., Teitelbaum, S. L., Ghiselli, R. & Gluck, S. Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* **245**, 855–857 (Aug. 1989) (cit. on p. 18).
61. Takahashi, N., Yamana, H., Yoshiki, S., Roodman, G. D., Mundy, G. R., Jones, S. J., Boyde, A. & Suda, T. Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinology* **122**, 1373–1382 (Apr. 1988) (cit. on p. 18).
62. Vaananen, H. K., Zhao, H., Mulari, M. & Halleen, J. M. The cell biology of osteoclast function. *Journal of Cell Science* **113**, 377–381 (Feb. 2000) (cit. on p. 18).
63. Chu, G. C.-Y., Zhau, H. E., Wang, R., Rogatko, A., Feng, X., Zayzafoon, M., Liu, Y., Farach-Carson, M. C., You, S., Kim, J., Freeman, M. R. & Chung, L. W. K. RANK- and c-Met-mediated signal network promotes prostate can-

- cer metastatic colonization. *Endocrine-Related Cancer* **21**, 311–326 (Apr. 2014) (cit. on pp. [19](#), [78](#)).
64. Keller, E. T. The role of osteoclastic activity in prostate cancer skeletal metastases. *Drugs of Today* **38**, 91–102 (Feb. 2002) (cit. on p. [19](#)).
65. Yin, J. J., Mohammad, K. S., Käkönen, S. M., Harris, S., Wu-Wong, J. R., Wessale, J. L., Padley, R. J., Garrett, I. R., Chirgwin, J. M. & Guise, T. A. A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. *Proceedings of the National Academy of Sciences* **100**, 10954–10959 (Sept. 2003) (cit. on pp. [19](#), [22](#)).
66. Weilbaecher, K. N., Guise, T. A. & McCauley, L. K. Cancer to bone: a fatal attraction. *Nature Reviews Cancer* **11**, 411–425 (June 2011) (cit. on p. [19](#)).
67. O’Shaughnessy, P. J., Baker, P., Sohnius, U., Haavisto, A.-M., Charlton, H. M. & Huhtaniemi, I. Fetal Development of Leydig Cell Activity in the Mouse Is Independent of Pituitary Gonadotroph Function. *Endocrinology* **139**, 1141–1146 (Mar. 1998) (cit. on p. [19](#)).
68. Jenster, G., Trapman, J. & Brinkmann, A. O. Nuclear import of the human androgen receptor. *Biochemical Journal* **293**, 761–768 (Aug. 1993) (cit. on p. [20](#)).
69. Faisst, S. & Meyer, S. Compilation of vertebrate-encoded transcription factors. *Nucleic Acids Research* **20**, 3–26 (Jan. 1992) (cit. on p. [20](#)).
70. Bolton, E. C., So, A. Y., Chaivorapol, C., Haqq, C. M., Li, H. & Yamamoto, K. R. Cell- and gene-specific regulation of primary target genes by the androgen receptor. *Genes & Development* **21**, 2005–2017 (Aug. 2007) (cit. on p. [20](#)).
71. Reddy, G. P. V., Barrack, E. R., Dou, Q. P., Menon, M., Pelley, R., Sarkar, F. H. & Sheng, S. Regulatory processes affecting androgen receptor expression, stability, and function: potential targets to treat hormone-refractory prostate cancer. *Journal of Cellular Biochemistry* **98**, 1408–1423 (Aug. 2006) (cit. on p. [20](#)).
72. Kotaja, N., Aittomäki, S., Silvennoinen, O., Palvimo, J. J. & Jänne, O. A. ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. *Molecular Endocrinology* **14**, 1986–2000 (Dec. 2000) (cit. on p. [21](#)).

73. Zhao, H., Dupont, J., Yakar, S., Karas, M. & LeRoith, D. PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGF-IR expression in prostate cancer cells. *Oncogene* **23**, 786–794 (Jan. 2004) (cit. on p. [21](#)).
74. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M. & Sellers, W. R. Loss of PTEN Expression in Paraffin-embedded Primary Prostate Cancer Correlates with High Gleason Score and Advanced Stage. *Cancer Research* **59**, 4291–4296 (Sept. 1999) (cit. on p. [21](#)).
75. 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. & Wu, H. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* **4**, 209–221 (Sept. 2003) (cit. on p. [21](#)).
76. Chen, G., Sircar, K., Aprikian, A., Potti, A., Goltzman, D. & Rabbani, S. A. Expression of RANKL/RANK/OPG in primary and metastatic human prostate cancer as markers of disease stage and functional regulation. *Cancer* **107**, 289–298 (July 2006) (cit. on p. [21](#)).
77. Garraway, I. P. Targeting the RANKL Pathway: Putting the Brakes on Prostate Cancer Progression in Bone. *Journal of Clinical Oncology* **31**, 3838–3840 (Oct. 2013) (cit. on p. [21](#)).
78. Jin, R., Sterling, J. A., Edwards, J. R., Degraff, D. J., Lee, C., Park, S. I. & Matusik, R. J. Activation of NF-kappa B Signaling Promotes Growth of Prostate Cancer Cells in Bone. *PLoS ONE* **8**, e60983 (2013) (cit. on p. [21](#)).
79. Odero-Marah, V. A., Wang, R., Chu, G., Zayzafoon, M., Xu, J., Shi, C., Marshall, F. F., Zhau, H. E. & Chung, L. W. Receptor activator of NF- κ B Ligand (RANKL) expression is associated with epithelial to mesenchymal transition in human prostate cancer cells. *Cell Research* **18**, 858–870 (2008) (cit. on pp. [21](#), [33](#), [38](#), [60](#), [78](#)).
80. Zhau, H. E., Odero-Marah, V., Lue, H.-W., Nomura, T., Wang, R., Chu, G., Liu, Z.-R., Zhou, B. P., Huang, W.-C. & Chung, L. W. K. Epithelial to mesenchymal transition (EMT) in human prostate cancer: lessons learned from ARCaP model. *Clinical & Experimental Metastasis* **25**, 601–610 (Oct. 2008) (cit. on pp. [21](#), [33](#), [57](#), [78](#)).
81. Roh, M. & Abdulkadir, S. A. Targeting the endothelin receptor in prostate cancer bone metastasis: back to the mouse? *Cancer biology & therapy* **9**, 615–617 (Apr. 2010) (cit. on p. [21](#)).

82. Nelson, J. B., Hedican, S. P., George, D. J., Reddi, A. H., Piantadosi, S., Eisenberger, M. A. & Simons, J. W. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nature Medicine* **1**, 944–949 (Sept. 1995) (cit. on p. [22](#)).
83. Akech, J., Wixted, J. J., Bedard, K., van der Deen, M., Hussain, S., Guise, T. A., van Wijnen, A. J., Stein, J. L., Languino, L. R., Altieri, D. C., Pratap, J., Keller, E., Stein, G. S. & Lian, J. B. Runx2 Association with Progression of Prostate Cancer in Patients: Mechanisms Mediating Bone Osteolysis and Osteoblastic Metastatic Lesions. *Oncogene* **29**, 811 (Feb. 2010) (cit. on pp. [22](#), [40](#), [54](#)).
84. Bryden, A. a. G., Islam, S., Freemont, A. J., Shanks, J. H., George, N. J. R. & Clarke, N. W. Parathyroid hormone-related peptide: expression in prostate cancer bone metastases. *Prostate Cancer and Prostatic Diseases* **5**, 59–62 (2002) (cit. on p. [22](#)).
85. Liao, J., Li, X., Koh, A. J., Berry, J. E., Thudi, N., Rosol, T. J., Pienta, K. J. & McCauley, L. K. Tumor expressed PTHrP facilitates prostate cancer-induced osteoblastic lesions. *International Journal of Cancer* **123**, 2267–2278 (Nov. 2008) (cit. on p. [22](#)).
86. Dougherty, K. M., Blomme, E. A. G., Koh, A. J., Henderson, J. E., Pienta, K. J., Rosol, T. J. & McCauley, L. K. Parathyroid Hormone-related Protein as a Growth Regulator of Prostate Carcinoma. *Cancer Research* **59**, 6015–6022 (Dec. 1999) (cit. on p. [22](#)).
87. Deftos, L. J., Barken, I., Burton, D. W., Hoffman, R. M. & Geller, J. Direct evidence that PTHrP expression promotes prostate cancer progression in bone. *Biochemical and Biophysical Research Communications* **327**, 468–472 (Feb. 2005) (cit. on p. [22](#)).
88. Pinzone, J. J., Hall, B. M., Thudi, N. K., Vonau, M., Qiang, Y.-W., Rosol, T. J. & Shaughnessy, J. D. The role of Dickkopf-1 in bone development, homeostasis, and disease. *Blood* **113**, 517–525 (Jan. 2009) (cit. on p. [23](#)).
89. Thudi, N. K., Martin, C. K., Murahari, S., Shu, S. T., Lanigan, L. G., Werbeck, J. L., Keller, E. T., McCauley, L. K., Pinzone, J. J. & Rosol, T. J. Dickkopf-1 (DKK-1) stimulated prostate cancer growth and metastasis and inhibited bone formation in osteoblastic bone metastases. *The Prostate* **71**, 615–625 (May 2011) (cit. on p. [23](#)).

90. Hall, C. L., Bafico, A., Dai, J., Aaronson, S. A. & Keller, E. T. Prostate Cancer Cells Promote Osteoblastic Bone Metastases through Wnts. *Cancer Research* **65**, 7554–7560 (Sept. 2005) (cit. on p. [23](#)).
91. Aalinkeel, R., Nair, B. B., Reynolds, J. L., Sykes, D. E., Mahajan, S. D., Chadha, K. C. & Schwartz, S. A. Overexpression of MMP-9 contributes to invasiveness of prostate cancer cell line LNCaP. *Immunological Investigations* **40**, 447–464 (2011) (cit. on p. [23](#)).
92. Gupta, A., Zhou, C. Q. & Chellaiah, M. A. Osteopontin and MMP9: Associations with VEGF Expression/Secretion and Angiogenesis in PC3 Prostate Cancer Cells. *Cancers* **5**, 617–638 (2013) (cit. on p. [23](#)).
93. Lynch, C. C., Hikosaka, A., Acuff, H. B., Martin, M. D., Kawai, N., Singh, R. K., Vargo-Gogola, T. C., Begtrup, J. L., Peterson, T. E., Fingleton, B., Shirai, T., Matrisian, L. M. & Futakuchi, M. MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. *Cancer Cell* **7**, 485–496 (May 2005) (cit. on p. [23](#)).
94. Li, Y. & Cozzi, P. J. Targeting uPA/uPAR in prostate cancer. *Cancer Treatment Reviews* **33**, 521–527 (Oct. 2007) (cit. on p. [24](#)).
95. Pulukuri, S. M., Gondi, C. S., Lakka, S. S., Jutla, A., Estes, N., Gujrati, M. & Rao, J. S. RNA Interference-directed Knockdown of Urokinase Plasminogen Activator and Urokinase Plasminogen Activator Receptor Inhibits Prostate Cancer Cell Invasion, Survival, and Tumorigenicity in Vivo. *Journal of Biological Chemistry* **280**, 36529–36540 (Oct. 2005) (cit. on p. [24](#)).
96. Shariat, S. F., Roehrborn, C. G., McConnell, J. D., Park, S., Alam, N., Wheeler, T. M. & Slawin, K. M. Association of the circulating levels of the urokinase system of plasminogen activation with the presence of prostate cancer and invasion, progression, and metastasis. *Journal of Clinical Oncology* **25**, 349–355 (Feb. 2007) (cit. on p. [24](#)).
97. McCabe, N. P., Angwafo, F. F., Zaher, A., Selman, S. H., Kouinche, A. & Jankun, J. Expression of soluble urokinase plasminogen activator receptor may be related to outcome in prostate cancer patients. *Oncology Reports* **7**, 879–961 (July 2000) (cit. on p. [24](#)).
98. Cozzi, P. J., Wang, J., Delprado, W., Madigan, M. C., Fairy, S., Russell, P. J. & Li, Y. Evaluation of urokinase plasminogen activator and its receptor in different grades of human prostate cancer. *Human Pathology* **37**, 1442–1451 (Nov. 2006) (cit. on p. [24](#)).

-
99. Di Lorenzo, G., Tortora, G., D'Armiento, F. P., De Rosa, G., Staibano, S., Autorino, R., D'Armiento, M., De Laurentiis, M., De Placido, S., Catalano, G., Bianco, A. R. & Ciardiello, F. Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clinical Cancer Research* **8**, 3438–3444 (Nov. 2002) (cit. on p. 25).
100. Cai, C. Q., Peng, Y., Buckley, M. T., Wei, J., Chen, F., Liebes, L., Gerald, W. L., Pincus, M. R., Osman, I. & Lee, P. Epidermal growth factor receptor activation in prostate cancer by three novel missense mutations. *Oncogene* **27**, 3201–3210 (Jan. 2008) (cit. on p. 25).
101. Sherwood, E. R., Van Dongen, J., Wood, C. G., Liao, S., Kozlowski, J. M. & Lee, C. Epidermal growth factor receptor activation in androgen-independent but not androgen-stimulated growth of human prostatic carcinoma cells. *British Journal of Cancer* **77**, 855–861 (Mar. 1998) (cit. on p. 25).
102. Kim, J.-H., Xu, C., Keum, Y.-S., Reddy, B., Conney, A. & Kong, A.-N. T. Inhibition of EGFR signaling in human prostate cancer PC-3 cells by combination treatment with β -phenylethyl isothiocyanate and curcumin. *Carcinogenesis* **27**, 475–482 (Mar. 2006) (cit. on p. 25).
103. Mellingerhoff, I. K., Vivanco, I., Kwon, A., Tran, C., Wongvipat, J. & Sawyers, C. L. HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell* **6**, 517–527 (Nov. 2004) (cit. on p. 25).
104. Wu, J. & Yu, E. Insulin-like growth factor receptor-1 (IGF-IR) as a target for prostate cancer therapy. *Cancer metastasis reviews* **33**, 607–617 (Sept. 2014) (cit. on p. 25).
105. Wolk, A., Mantzoros, C. S., Andersson, S.-O., Bergström, R., Signorello, L. B., Laggiou, P., Adami, H.-O. & Trichopoulos, D. Insulin-Like Growth Factor 1 and Prostate Cancer Risk: A Population-Based, Case-Control Study. *Journal of the National Cancer Institute* **90**, 911–915 (June 1998) (cit. on p. 25).
106. Zu, K., Martin, N. E., Fiorentino, M., Flavin, R., Lis, R. T., Sinnott, J. A., Finn, S., Penney, K. L., Ma, J., Fazli, L., Gleave, M. E., Bismar, T. A., Stampfer, M. J., Pollak, M. N., Loda, M., Mucci, L. A. & Giovannucci, E. Protein expression of PTEN, insulin-like growth factor I receptor (IGF-IR), and lethal prostate cancer: a prospective study. *Cancer Epidemiology, Biomarkers & Prevention* **22**, 1984–1993 (Nov. 2013) (cit. on p. 25).

107. Hellawell, G. O., Turner, G. D. H., Davies, D. R., Poulsom, R., Brewster, S. F. & Macaulay, V. M. Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. *Cancer Research* **62**, 2942–2950 (May 2002) (cit. on p. 25).
108. Sayeed, A., Fedele, C., Trerotola, M., Ganguly, K. K. & Languino, L. R. IGF-IR Promotes Prostate Cancer Growth by Stabilizing $\alpha 5\beta 1$ Integrin Protein Levels. *PLoS ONE* **8**, e76513 (Oct. 2013) (cit. on p. 25).
109. Verras, M. & Sun, Z. Beta-catenin is involved in insulin-like growth factor 1-mediated transactivation of the androgen receptor. *Molecular Endocrinology* **19**, 391–398 (Feb. 2005) (cit. on p. 26).
110. Wu, J. D., Haugk, K., Woodke, L., Nelson, P., Coleman, I. & Plymate, S. R. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. *Journal of Cellular Biochemistry* **99**, 392–401 (Oct. 2006) (cit. on p. 26).
111. Wang, L.-N., Chen, W.-W., Zhang, J., Li, C.-Y., Liu, C.-Y., Xue, J., Zhang, P.-J. & Jiang, A.-L. The miRNA let-7a1 inhibits the expression of insulin-like growth factor 1 receptor (IGF1R) in prostate cancer PC-3 cells. *Asian Journal of Andrology* **15**, 753–758 (Nov. 2013) (cit. on p. 26).
112. Nadiminty, N., Tummala, R., Lou, W., Zhu, Y., Shi, X.-B., Zou, J. X., Chen, H., Zhang, J., Chen, X., Luo, J., deVere White, R. W., Kung, H.-J., Evans, C. P. & Gao, A. C. MicroRNA let-7c Is Downregulated in Prostate Cancer and Suppresses Prostate Cancer Growth. *PLoS ONE* **7**, e32832 (Mar. 2012) (cit. on p. 26).
113. Rochester, M. A., Riedemann, J., Hellawell, G. O., Brewster, S. F. & Macaulay, V. M. Silencing of the IGF1R gene enhances sensitivity to DNA-damaging agents in both PTEN wild-type and mutant human prostate cancer. *Cancer Gene Therapy* **12**, 90–100 (Jan. 2005) (cit. on p. 26).
114. Goel, H. L. & Mercurio, A. M. VEGF targets the tumour cell. *Nature Reviews Cancer* **13**, 871–882 (Dec. 2013) (cit. on p. 26).
115. Harper, M. E., Glynne-Jones, E., Goddard, L., Thurston, V. J. & Griffiths, K. Vascular endothelial growth factor (VEGF) expression in prostatic tumours and its relationship to neuroendocrine cells. *British Journal of Cancer* **74**, 910–916 (Sept. 1996) (cit. on p. 26).

116. Jackson, M. W., Bentel, J. M. & Tilley, W. D. Vascular endothelial growth factor (VEGF) expression in prostate cancer and benign prostatic hyperplasia. *The Journal of Urology* **157**, 2323–2328 (June 1997) (cit. on p. 26).
117. Ferrer, F. A., Miller, L. J., Andrawis, R. I., Kurtzman, S. H., Albertsen, P. C., Laudone, V. P. & Kreutzer, D. L. Vascular endothelial growth factor (VEGF) expression in human prostate cancer: in situ and in vitro expression of VEGF by human prostate cancer cells. *The Journal of Urology* **157**, 2329–2333 (June 1997) (cit. on p. 26).
118. Kirschenbaum, A., Wang, J. P., Ren, M., Schiff, J. D., Aaronson, S. A., Droller, M. J., Ferrara, N., Holland, J. F. & Levine, A. C. Inhibition of vascular endothelial cell growth factor suppresses the in vivo growth of human prostate tumors. *Urologic Oncology* **3**, 3–10 (Feb. 1997) (cit. on p. 26).
119. Dai, J., Kitagawa, Y., Zhang, J., Yao, Z., Mizokami, A., Cheng, S., Nör, J., McCauley, L. K., Taichman, R. S. & Keller, E. T. Vascular Endothelial Growth Factor Contributes to the Prostate Cancer-Induced Osteoblast Differentiation Mediated by Bone Morphogenetic Protein. *Cancer Research* **64**, 994–999 (Feb. 2004) (cit. on p. 26).
120. Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J. & Nohe, A. Bone Morphogenetic Proteins: A critical review. *Cellular Signalling* **23**, 609–620 (Apr. 2011) (cit. on p. 27).
121. Hamdy, F. C., Autzen, P., Robinson, M. C., Horne, C. H. W., Neal, D. E. & Robson, C. N. Immunolocalization and Messenger RNA Expression of Bone Morphogenetic Protein-6 in Human Benign and Malignant Prostatic Tissue. *Cancer Research* **57**, 4427–4431 (Oct. 1997) (cit. on p. 27).
122. Grishina, I. B., Kim, S. Y., Ferrara, C., Makarenkova, H. P. & Walden, P. D. BMP7 inhibits branching morphogenesis in the prostate gland and interferes with Notch signaling. *Developmental Biology* **288**, 334–347 (Dec. 2005) (cit. on p. 27).
123. Buijs, J. T., Rentsch, C. A., van der Horst, G., van Overveld, P. G. M., Wetterwald, A., Schwaninger, R., Henriquez, N. V., Ten Dijke, P., Borovecki, F., Markwalder, R., Thalmann, G. N., Papapoulos, S. E., Pelger, R. C. M., Vukicevic, S., Cecchini, M. G., Löwik, C. W. G. M. & van der Pluijm, G. BMP7, a putative regulator of epithelial homeostasis in the human prostate, is a potent inhibitor of prostate cancer bone metastasis in vivo. *The American Journal of Pathology* **171**, 1047–1057 (Sept. 2007) (cit. on p. 27).

-
124. Lim, M., Chuong, C.-M. & Roy-Burman, P. PI3K, Erk signaling in BMP7-induced epithelial-mesenchymal transition (EMT) of PC-3 prostate cancer cells in 2- and 3-dimensional cultures. *Hormones & Cancer* **2**, 298–309 (Oct. 2011) (cit. on p. 27).
125. Morrissey, C., Brown, L. G., Pitts, T. E., Vessella, R. L. & Corey, E. 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**, 192–205 (Feb. 2010) (cit. on p. 27).
126. Onder, T. T., Gupta, P. B., Mani, S. A., Yang, J., Lander, E. S. & Weinberg, R. A. Loss of E-Cadherin Promotes Metastasis via Multiple Downstream Transcriptional Pathways. *Cancer Research* **68**, 3645–3654 (May 2008) (cit. on p. 28).
127. Zeisberg, M. & Neilson, E. G. Biomarkers for epithelial-mesenchymal transitions. *The Journal of Clinical Investigation* **119**, 1429–1437 (June 2009) (cit. on p. 28).
128. Aigner, K., Dampier, B., Descovich, L., Mikula, M., Sultan, A., Schreiber, M., Mikulits, W., Brabletz, T., Strand, D., Obrist, P., Sommergruber, W., Schweifer, N., Wernitznig, A., Beug, H., Foisner, R. & Eger, A. The transcription factor ZEB1 (δ EF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. *Oncogene* **26**, 6979–6988 (May 2007) (cit. on p. 28).
129. Vandewalle, C., Comijn, J., Craene, B. D., Vermassen, P., Bruyneel, E., Andersen, H., Tulchinsky, E., Roy, F. V. & Bex, G. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. *Nucleic Acids Research* **33**, 6566–6578 (Jan. 2005) (cit. on p. 28).
130. Cano, A., Pérez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F. & Nieto, M. A. The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature Cell Biology* **2**, 76–83 (Feb. 2000) (cit. on p. 28).
131. Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.-R., Zhang, Y. & Wrana, J. L. Regulation of the Polarity Protein Par6 by TGF β Receptors Controls Epithelial Cell Plasticity. *Science* **307**, 1603–1609 (Mar. 2005) (cit. on p. 28).
132. Lamouille, S., Xu, J. & Derynck, R. Molecular mechanisms of epithelial–mesenchymal transition. *Nature Reviews Molecular Cell Biology* **15**, 178–196 (Mar. 2014) (cit. on p. 28).

133. Acloque, H., Adams, M. S., Fishwick, K., Bronner-Fraser, M. & Nieto, M. A. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *Journal of Clinical Investigation* **119**, 1438–1449 (June 2009) (cit. on pp. 28, 31).
134. Mani, S. A., Guo, W., Liao, M.-J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Briskin, C., Yang, J. & Weinberg, R. A. The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells. *Cell* **133**, 704–715 (May 2008) (cit. on p. 28).
135. Nakaya, Y., Sukowati, E. W., Wu, Y. & Sheng, G. RhoA and microtubule dynamics control cell-basement membrane interaction in EMT during gastrulation. *Nature Cell Biology* **10**, 765–775 (July 2008) (cit. on p. 28).
136. Fantozzi, A., Gruber, D. C., Pisarsky, L., Heck, C., Kunita, A., Yilmaz, M., Meyer-Schaller, N., Cornille, K., Hopfer, U., Bentires-Alj, M. & Christofori, G. VEGF-mediated angiogenesis links EMT-induced cancer stemness to tumor initiation. *Cancer Research* **74**, 1566–1575 (Mar. 2014) (cit. on p. 29).
137. Nieto, M. A. The snail superfamily of zinc-finger transcription factors. *Nature Reviews Molecular Cell Biology* **3**, 155–166 (Mar. 2002) (cit. on p. 30).
138. Van Roy, F. & Berx, G. The cell-cell adhesion molecule E-cadherin. *Cellular and molecular life sciences* **65**, 3756–3788 (Nov. 2008) (cit. on p. 30).
139. Miyazono, K. Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences* **85**, 314–323 (2009) (cit. on p. 31).
140. Jiang, Y.-G., Luo, Y., He, D.-l., Li, X., Zhang, L.-l., Peng, T., Li, M.-C. & Lin, Y.-H. Role of Wnt/beta-catenin signaling pathway in epithelial-mesenchymal transition of human prostate cancer induced by hypoxia-inducible factor-1alpha. *International Journal of Urology* **14**, 1034–1039 (Nov. 2007) (cit. on p. 31).
141. Jossion, S., Sharp, S., Sung, S.-Y., Johnstone, P. A. S., Aneja, R., Wang, R., Gururajan, M., Turner, T., Chung, L. W. K. & Yates, C. Tumor-Stromal Interactions Influence Radiation Sensitivity in Epithelial- versus Mesenchymal-Like Prostate Cancer Cells. *Journal of Oncology* **2010** (2010) (cit. on p. 32).
142. Van der Pluijm, G. Epithelial plasticity, cancer stem cells and bone metastasis formation. *Bone* **48**, 37–43 (Jan. 2011) (cit. on p. 32).

143. Zhau, H. Y. E., Chang, S.-M., Chen, B.-Q., Wang, Y., Zhang, H., Kao, C., Sang, Q. A., Pathak, S. J. & Chung, L. W. K. Androgen-repressed phenotype in human prostate cancer. *Proceedings of the National Academy of Sciences* **93**, 15152–15157 (Dec. 1996) (cit. on pp. [33](#), [60](#)).
144. Cinar, B., Koeneman, K. S., Edlund, M., Prins, G. S., Zhau, H. E. & Chung, L. W. K. Androgen Receptor Mediates the Reduced Tumor Growth, Enhanced Androgen Responsiveness, and Selected Target Gene Transactivation in a Human Prostate Cancer Cell Line. *Cancer Research* **61**, 7310–7317 (Oct. 2001) (cit. on p. [33](#)).
145. Xu, J., Wang, R., Xie, Z. H., Odero-Marah, V., Pathak, S., Multani, A., Chung, L. W. K. & Zhau, H. E. Prostate cancer metastasis: Role of the host microenvironment in promoting epithelial to mesenchymal transition and increased bone and adrenal gland metastasis. *The Prostate* **66**, 1664–1673 (Nov. 2006) (cit. on pp. [33](#), [79](#)).
146. Zhu, G., Zhou, J., Song, W., Wu, D., Dang, Q., Zhang, L., Li, L., Wang, X. & He, D. Role of GLI-1 in epidermal growth factor-induced invasiveness of ARCaPE prostate cancer cells. *Oncology Reports* **30**, 904–910 (Aug. 2013) (cit. on p. [33](#)).
147. Graham, T. R., Zhau, H. E., Odero-Marah, V. A., Osunkoya, A. O., Kimbro, K. S., Tighiouart, M., Liu, T., Simons, J. W. & O'Regan, R. M. Insulin-like Growth Factor-I-Dependent Up-regulation of ZEB1 Drives Epithelial-to-Mesenchymal Transition in Human Prostate Cancer Cells. *Cancer Research* **68**, 2479–2488 (Apr. 2008) (cit. on p. [33](#)).
148. He, H., Yang, X., Davidson, A. J., Wu, D., Marshall, F. F., Chung, L. W. K., Zhau, H. E. & Wang, R. Progressive epithelial to mesenchymal transitions in ARCaPE prostate cancer cells during xenograft tumor formation and metastasis. *The Prostate* **70**, 518–528 (Apr. 2010) (cit. on p. [34](#)).
149. He, H., Davidson, A. J., Wu, D., Marshall, F. F., Chung, L. W. K., Zhau, H. E., He, D. & Wang, R. Phorbol ester phorbol-12-myristate-13-acetate induces epithelial to mesenchymal transition in human prostate cancer ARCaPE cells. *The Prostate* **70**, 1119–1126 (July 2010) (cit. on p. [34](#)).
150. Lue, H.-W., Yang, X., Wang, R., Qian, W., Xu, R. Z. H., Lyles, R., Osunkoya, A. O., Zhou, B. P., Vessella, R. L., Zayzafoon, M., Liu, Z.-R., Zhau, H. E. & Chung, L. W. K. LIV-1 Promotes Prostate Cancer Epithelial-to-Mesenchymal Transition and Metastasis through HB-EGF Shedding and EGFR-Mediated ERK Signaling. *PLoS ONE* **6**, e27720 (Nov. 2011) (cit. on p. [34](#)).

151. Jossan, S., Nomura, T., Lin, J.-T., Huang, W.-C., Wu, D., Zhau, H. E., Zayzafoon, M., Weizmann, M. N., Gururajan, M. & Chung, L. W. K. β 2-Microglobulin Induces Epithelial to Mesenchymal Transition and Confers Cancer Lethality and Bone Metastasis in Human Cancer Cells. *Cancer Research* **71**, 2600–2610 (Apr. 2011) (cit. on pp. 34, 78).
152. Lindow, M. & Kauppinen, S. Discovering the first microRNA-targeted drug. *The Journal of Cell Biology* **199**, 407–412 (Oct. 2012) (cit. on p. 34).
153. Ha, M. & Kim, V. N. Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology* **15**, 509–524 (Aug. 2014) (cit. on p. 35).
154. MacFarlane, L.-A. & Murphy, P. R. MicroRNA: Biogenesis, Function and Role in Cancer. *Current Genomics* **11**, 537–561 (Nov. 2010) (cit. on pp. 36, 104).
155. Pang, Y., Young, C. Y. F. & Yuan, H. MicroRNAs and prostate cancer. *Acta Biochimica et Biophysica Sinica* **42**, 363–369 (June 2010) (cit. on p. 36).
156. Fang, Y.-X. & Gao, W.-Q. Roles of microRNAs during prostatic tumorigenesis and tumor progression. *Oncogene* **33**, 135–147 (Jan. 2014) (cit. on p. 36).
157. Maugeri-Sacca, M., Coppola, V., De Maria, R. & Bonci, D. Functional role of microRNAs in prostate cancer and therapeutic opportunities. *Critical Reviews in Oncogenesis* **18**, 303–315 (2013) (cit. on pp. 36, 104).
158. Sita-Lumsden, A., Dart, D. A., Waxman, J. & Bevan, C. L. Circulating microRNAs as potential new biomarkers for prostate cancer. *British Journal of Cancer* (Apr. 2013) (cit. on pp. 36, 104).
159. Bryant, R. J., Pawlowski, T., Catto, J. W. F., Marsden, G., Vessella, R. L., Rhee, B., Kuslich, C., Visakorpi, T. & Hamdy, F. C. Changes in circulating microRNA levels associated with prostate cancer. *British journal of cancer* **106**, 768–774 (Feb. 2012) (cit. on pp. 36, 104).
160. Banyard, J., Chung, I., Wilson, A. M., Vetter, G., Le Béchec, A., Bielenberg, D. R. & Zetter, B. R. Regulation of epithelial plasticity by miR-424 and miR-200 in a new prostate cancer metastasis model. *Scientific Reports* **3** (Nov. 2013) (cit. on p. 36).
161. Williams, L. V., Veliceasa, D., Vinokour, E. & Volpert, O. V. miR-200b Inhibits Prostate Cancer EMT, Growth and Metastasis. *PLoS ONE* **8**, e83991 (Dec. 2013) (cit. on p. 36).

162. Hill, L., Browne, G. & Tulchinsky, E. ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. *International Journal of Cancer* **132**, 745–754 (Feb. 2013) (cit. on p. 36).
163. Saini, S., Majid, S., Yamamura, S., Tabatabai, Z. L., Suh, S. O., Shahryari, V., Chen, Y., Deng, G., Tanaka, Y. & Dahiya, R. Regulatory role of miR-203 in prostate cancer progression and metastasis. *Clinical Cancer Research* (Dec. 2010) (cit. on pp. 36, 105, 107, 124).
164. Craene, B. D. & Berx, G. Regulatory networks defining EMT during cancer initiation and progression. *Nature Reviews Cancer* **13**, 97–110 (Feb. 2013) (cit. on p. 36).
165. Tucci, P., Agostini, M., Grespi, F., Markert, E. K., Terrinoni, A., Vousden, K. H., Muller, P. A. J., Dötsch, V., Kehrloesser, S., Sayan, B. S., Giaccone, G., Lowe, S. W., Takahashi, N., Vandenabeele, P., Knight, R. A., Levine, A. J. & Melino, G. Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. *Proceedings of the National Academy of Sciences* **109**, 15312–15317 (Sept. 2012) (cit. on p. 36).
166. Singh, A. S. & Figg, W. D. In vivo models of prostate cancer metastasis to bone. *The Journal of Urology* **174**, 820–826 (Sept. 2005) (cit. on p. 38).
167. Henry, M. D., Silva, M. D., Wen, S., Siebert, E., Solin, E., Chandra, S. & Worland, P. J. Spiculated periosteal response induced by intraosseous injection of 22Rv1 prostate cancer cells resembles subset of bone metastases in prostate cancer patients. *The Prostate* **65**, 347–354 (Dec. 2005) (cit. on pp. 38, 60, 79).
168. Simmons, J. K., Elshafae, S. M., Keller, E. T., McCauley, L. K. & Rosol, T. J. Review of Animal Models of Prostate Cancer Bone Metastasis. *Veterinary Sciences* **1**, 16–39 (June 2014) (cit. on pp. 39, 60).
169. Drake, J. M., Gabriel, C. L. & Henry, M. D. Assessing Tumor Growth and Distribution in a Model of Prostate Cancer Metastasis using Bioluminescence Imaging. *Clinical & Experimental Metastasis* **22**, 674–684 (Dec. 2005) (cit. on pp. 38, 60).
170. Dranoff, G. Experimental mouse tumour models: what can be learnt about human cancer immunology? *Nature Reviews Immunology* **12**, 61–66 (Jan. 2012) (cit. on p. 38).
171. Koenen, K. S., Yeung, F. & Chung, L. W. Osteomimetic properties of prostate cancer cells: A hypothesis supporting the predilection of prostate

- cancer metastasis and growth in the bone environment. *The Prostate* **39**, 246–261 (1999) (cit. on pp. 40, 53, 128).
172. Rucci, N. & Teti, A. Osteomimicry: how tumor cells try to deceive the bone. *Frontiers in bioscience (Scholar edition)* **2**, 907–915 (2010) (cit. on pp. 40, 128).
173. Pratap, J., Lian, J. B., Javed, A., Barnes, G. L., van Wijnen, A. J., Stein, J. L. & Stein, G. S. Regulatory roles of Runx2 in metastatic tumor and cancer cell interactions with bone. *Cancer Metastasis Reviews* **25**, 589–600 (Dec. 2006) (cit. on p. 40).
174. Desai, B., Rogers, M. J. & Chellaiah, M. A. Mechanisms of osteopontin and CD44 as metastatic principles in prostate cancer cells. *Molecular Cancer* **6**, 18 (Mar. 2007) (cit. on pp. 40, 54).
175. Robertson, B. W. & Chellaiah, M. A. Osteopontin induces beta-catenin signaling through activation of Akt in prostate cancer cells. *Experimental Cell Research* **316**, 1–11 (Jan. 2010) (cit. on pp. 40, 54).
176. Khodavirdi, A. C., Song, Z., Yang, S., Zhong, C., Wang, S., Wu, H., Pritchard, C., Nelson, P. S. & Roy-Burman, P. Increased expression of osteopontin contributes to the progression of prostate cancer. *Cancer Research* **66**, 883–888 (Jan. 2006) (cit. on pp. 40, 54).
177. Robertson, B. W., Bonsal, L. & Chellaiah, M. A. Regulation of Erk1/2 activation by osteopontin in PC3 human prostate cancer cells. *Molecular Cancer* **9**, 260 (Sept. 2010) (cit. on pp. 40, 54).
178. Thoms, J. W., Dal Pra, A., Anborgh, P. H., Christensen, E., Fleshner, N., Menard, C., Chadwick, K., Milosevic, M., Catton, C., Pintilie, M., Chambers, A. F. & Bristow, R. G. Plasma osteopontin as a biomarker of prostate cancer aggression: relationship to risk category and treatment response. *British Journal of Cancer* **107**, 840–846 (Aug. 2012) (cit. on pp. 40, 54).
179. Gardner, T. A., Lee, S.-J., Lee, S.-D., Li, X., Shirakawa, T., Kwon, D. D., Park, R. Y., Ahn, K. Y. & Jung, C. Differential expression of osteocalcin during the metastatic progression of prostate cancer. *Oncology Reports* **21**, 903–908 (Apr. 2009) (cit. on pp. 40, 54).
180. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**, 671–675 (July 2012) (cit. on p. 49).

181. Brubaker, K. D., Vessella, R. L., Brown, L. G. & Corey, E. Prostate cancer expression of runt-domain transcription factor Runx2, a key regulator of osteoblast differentiation and function. *The Prostate* **56**, 13–22 (June 2003) (cit. on p. 54).
182. Carlinfante, G., Vassiliou, D., Svensson, O., Wendel, M., Heinegård, D. & Andersson, G. Differential expression of osteopontin and bone sialoprotein in bone metastasis of breast and prostate carcinoma. *Clinical & Experimental Metastasis* **20**, 437–444 (2003) (cit. on p. 54).
183. Waltregny, D., Bellahcène, A., de Leval, X., Florkin, B., Weidle, U. & Castronovo, V. Increased expression of bone sialoprotein in bone metastases compared with visceral metastases in human breast and prostate cancers. *Journal of Bone and Mineral Research* **15**, 834–843 (May 2000) (cit. on p. 54).
184. Huang, W.-C., Xie, Z., Konaka, H., Sodek, J., Zhau, H. E. & Chung, L. W. K. Human osteocalcin and bone sialoprotein mediating osteomimicry of prostate cancer cells: role of cAMP-dependent protein kinase A signaling pathway. *Cancer research* **65**, 2303–2313 (Mar. 2005) (cit. on p. 54).
185. Thomas, R., True, L. D., Bassuk, J. A., Lange, P. H. & Vessella, R. L. Differential Expression of Osteonectin/SPARC during Human Prostate Cancer Progression. *Clinical Cancer Research* **6**, 1140–1149 (Mar. 2000) (cit. on p. 54).
186. Jacob, K., Webber, M., Benayahu, D. & Kleinman, H. K. Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. *Cancer Research* **59**, 4453–4457 (Sept. 1999) (cit. on p. 54).
187. Kapinas, K., Lowther, K. M., Kessler, C. B., Tilbury, K., Lieberman, J. R., Tirnauer, J. S., Campagnola, P. & Delany, A. M. Bone matrix osteonectin limits prostate cancer cell growth and survival. *Matrix Biology* **31**, 299–307 (June 2012) (cit. on p. 54).
188. Hall, C. L., Dai, J., van Golen, K. L., Keller, E. T. & Long, M. W. Type I collagen receptor (alpha 2 beta 1) signaling promotes the growth of human prostate cancer cells within the bone. *Cancer Research* **66**, 8648–8654 (Sept. 2006) (cit. on p. 54).
189. Hall, C. L., Dubyk, C. W., Riesenberger, T. A., Shein, D., Keller, E. T. & van Golen, K. L. Type I Collagen Receptor ($\alpha_2\beta_1$) Signaling Promotes

- Prostate Cancer Invasion through RhoC GTPase. *Neoplasia* **10**, 797–803 (Aug. 2008) (cit. on p. 54).
190. Lin, D. L., Tarnowski, C. P., Zhang, J., Dai, J., Rohn, E., Patel, A. H., Morris, M. D. & Keller, E. T. Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes in vitro. *The Prostate* **47**, 212–221 (May 2001) (cit. on pp. 54, 64, 79).
 191. Zhau, H. E., He, H., Wang, C. Y., Zayzafoon, M., Morrissey, C., Vessella, R. L., Marshall, F. F., Chung, L. W. K. & Wang, R. Human Prostate Cancer Harbors the Stem Cell Properties of Bone Marrow Mesenchymal Stem Cells. *Clinical Cancer Research* **17**, 2159–2169 (Apr. 2011) (cit. on pp. 55, 64).
 192. Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., Byrne, C. J., Heuer, M. L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A. P., Sander, C. & Schultz, N. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discovery* **2**, 401–404 (May 2012) (cit. on pp. 56, 75).
 193. R Core Team. *R: A Language and Environment for Statistical Computing* ISBN 3-900051-07-0. R Foundation for Statistical Computing (Vienna, Austria, 2013) (cit. on p. 56).
 194. Taylor, R. Interpretation of the Correlation Coefficient: A Basic Review. *Journal of Diagnostic Medical Sonography* **6**, 35–39 (Jan. 1990) (cit. on p. 56).
 195. Ghosh-Choudhury, N., Windle, J. J., Koop, B. A., Harris, M. A., Guerrero, D. L., Wozney, J. M., Mundy, G. R. & Harris, S. E. Immortalized murine osteoblasts derived from BMP 2-T-antigen expressing transgenic mice. *Endocrinology* **137**, 331–339 (Jan. 1996) (cit. on p. 58).
 196. Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A. A., Kim, S., Wilson, C. J., Lehár, J., Kryukov, G. V., Sonkin, D., Reddy, A., Liu, M., Murray, L., Berger, M. F., Monahan, J. E., Morais, P., Meltzer, J., Korejwa, A., Jané-Valbuena, J., Mapa, F. A., Thibault, J., Bric-Furlong, E., Raman, P., Shipway, A., Engels, I. H., Cheng, J., Yu, G. K., Yu, J., Aspesi, P., de Silva, M., Jagtap, K., Jones, M. D., Wang, L., Hatton, C., Palessandolo, E., Gupta, S., Mahan, S., Sougnez, C., Onofrio, R. C., Liefeld, T., MacConaill, L., Winckler, W., Reich, M., Li, N., Mesirov, J. P., Gabriel, S. B., Getz, G., Ardlie, K., Chan, V., Myer, V. E., Weber, B. L., Porter, J., Warmuth, M., Finan, P., Harris, J. L., Meyerson, M., Golub, T. R., Morrissey, M. P., Sellers, W. R., Schlegel, R. & Garraway, L. A. The Cancer Cell

- Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603–607 (Mar. 2012) (cit. on p. 59).
197. Sobel, R. & Sadar, M. Cell lines used in prostate cancer research: a compendium of old and new lines—part 1. *The Journal of Urology* **173**, 342–359 (Feb. 2005) (cit. on p. 60).
 198. Sobel, R. & Sadar, M. Cell lines used in prostate cancer research: a compendium of old and new lines—part 2. *The Journal of Urology* **173**, 360–372 (Feb. 2005) (cit. on p. 60).
 199. Li, X., Loberg, R., Liao, J., Ying, C., Snyder, L. A., Pienta, K. J. & McCauley, L. K. A Destructive Cascade Mediated by CCL2 Facilitates Prostate Cancer Growth in Bone. *Cancer research* **69**, 1685–1692 (Feb. 2009) (cit. on p. 60).
 200. Yin, J., Pollock, C., Tracy, K., Chock, M., Martin, P., Oberst, M. & Kelly, K. Activation of the RalGEF/Ral Pathway Promotes Prostate Cancer Metastasis to Bone. *Molecular and Cellular Biology* **27**, 7538–7550 (Nov. 2007) (cit. on p. 60).
 201. Goya, M., Miyamoto, S., Nagai, K., Ohki, Y., Nakamura, K., Shitara, K., Maeda, H., Sangai, T., Kodama, K., Endoh, Y., Ishii, G., Hasebe, T., Yonou, H., Hatano, T., Ogawa, Y. & Ochiai, A. Growth inhibition of human prostate cancer cells in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice by a ligand-specific antibody to human insulin-like growth factors. *Cancer Research* **64**, 6252–6258 (Sept. 2004) (cit. on p. 60).
 202. Garba, M. T. & Marie, P. J. Alkaline phosphatase inhibition by levamisole prevents 1,25-dihydroxyvitamin D3-stimulated bone mineralization in the mouse. *Calcified Tissue International* **38**, 296–302 (May 1986) (cit. on p. 71).
 203. 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. Integrative Genomic Profiling of Human Prostate Cancer. *Cancer Cell* **18**, 11–22 (July 2010) (cit. on pp. 75, 81).
 204. Wu, T. T., Sikes, R. A., Cui, Q., Thalmann, G. N., Kao, C., Murphy, C. F., Yang, H., Zhau, H. E., Balian, G. & Chung, L. W. Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by

- prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *International journal of cancer* **77**, 887–894 (Sept. 1998) (cit. on p. 79).
205. Fradet, A., Sorel, H., Depalle, B., Serre, C. M., Farlay, D., Turtoi, A., Belahcene, A., Follet, H., Castronovo, V., Clézardin, P. & Bonnelye, E. A New Murine Model of Osteoblastic/Osteolytic Lesions from Human Androgen-Resistant Prostate Cancer. *PLoS ONE* **8**, e75092 (Sept. 2013) (cit. on p. 79).
 206. Millan, J. L. Alkaline Phosphatases. *Purinergic Signalling* **2**, 335–341 (June 2006) (cit. on p. 80).
 207. Fang, W. G., Pirnia, F., Bang, Y. J., Myers, C. E. & Trepel, J. B. P2-purinergic receptor agonists inhibit the growth of androgen-independent prostate carcinoma cells. *Journal of Clinical Investigation* **89**, 191–196 (Jan. 1992) (cit. on pp. 80, 85, 99).
 208. Díez-Zaera, M., Díaz-Hernández, J. I., Hernández-Álvarez, E., Zimmermann, H., Díaz-Hernández, M. & Miras-Portugal, M. T. Tissue-nonspecific alkaline phosphatase promotes axonal growth of hippocampal neurons. *Molecular Biology of the Cell* **22**, 1014–1024 (Apr. 2011) (cit. on p. 80).
 209. Street, S. E., Kramer, N. J., Walsh, P. L., Taylor-Blake, B., Yadav, M. C., King, I. F., Vihko, P., Wightman, R. M., Millán, J. L. & Zylka, M. J. Tissue-Nonspecific Alkaline Phosphatase Acts Redundantly with PAP and NT5E to Generate Adenosine in the Dorsal Spinal Cord. *The Journal of Neuroscience* **33**, 11314–11322 (July 2013) (cit. on p. 80).
 210. Cunha, G. R., Fujii, H., Neubauer, B. L., Shannon, J. M., Sawyer, L. & Reese, B. A. Epithelial-mesenchymal interactions in prostatic development. I. morphological observations of prostatic induction by urogenital sinus mesenchyme in epithelium of the adult rodent urinary bladder. *The Journal of Cell Biology* **96**, 1662–1670 (June 1983) (cit. on p. 80).
 211. Falzoni, S., Donvito, G. & Virgilio, F. D. Detecting adenosine triphosphate in the pericellular space. *Interface Focus* **3**, 20120101 (June 2013) (cit. on pp. 83, 88, 100).
 212. Burnstock, G. Purinergic signalling. *British Journal of Pharmacology* **147**, S172–S181 (Jan. 2006) (cit. on pp. 84, 95).
 213. Burnstock, G., Arnett, T. R. & Orriss, I. R. Purinergic signalling in the musculoskeletal system. *Purinergic Signalling* **9**, 541–572 (Dec. 2013) (cit. on p. 84).

-
214. Jiang, L.-H. P2X receptor-mediated ATP purinergic signaling in health and disease. *Cell Health and Cytoskeleton*, 83 (Sept. 2012) (cit. on p. 84).
215. Linden, J. Structure and function of A1 adenosine receptors. *FASEB journal* **5**, 2668–2676 (Sept. 1991) (cit. on p. 84).
216. Roger, S. & Pelegrin, P. P2X7 receptor antagonism in the treatment of cancers. *Expert Opinion on Investigational Drugs* **20**, 875–880 (July 2011) (cit. on p. 85).
217. Adinolfi, E., Raffaghello, L., Giuliani, A. L., Cavazzini, L., Capece, M., Chiozzi, P., Bianchi, G., Kroemer, G., Pistoia, V. & Virgilio, F. D. Expression of P2X7 Receptor Increases In Vivo Tumor Growth. *Cancer Research* **72**, 2957–2969 (June 2012) (cit. on p. 85).
218. Gorodeski, G. I. P2X7-mediated chemoprevention of epithelial cancers. *Expert Opinion on Therapeutic Targets* **13**, 1313–1332 (Oct. 2009) (cit. on p. 85).
219. Barden, J. A., Yuksel, A., Pedersen, J., Danieletto, S. & Warick, D. Non-Functional P2X7: A Novel and Ubiquitous Target in Human Cancer. *Journal of Clinical and Cellular Immunology* **5**, 237 (2014) (cit. on p. 85).
220. Slater, M., Danieletto, S., Gidley-Baird, A., Teh, L. C. & Barden, J. A. Early prostate cancer detected using expression of non-functional cytolytic P2X7 receptors. *Histopathology* **44**, 206–215 (Mar. 2004) (cit. on p. 85).
221. Ghalali, A., Ye, Z.-w., Högberg, J. & Stenius, U. Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN) and PH Domain and Leucine-rich Repeat Phosphatase Cross-talk (PHLPP) in Cancer Cells and in Transforming Growth Factor β -Activated Stem Cells. *Journal of Biological Chemistry* **289**, 11601–11615 (Apr. 2014) (cit. on p. 85).
222. Ghalali, A., Wiklund, F., Zheng, H., Stenius, U. & Högberg, J. Atorvastatin prevents ATP-driven invasiveness via P2X7 and EHBP1 signaling in PTEN-expressing prostate cancer cells. *Carcinogenesis* **35**, 1547–1555 (July 2014) (cit. on p. 85).
223. Zhang, Y., Gong, L.-h., Zhang, H.-q., Du, Q., You, J.-f., Tian, X.-x. & Fang, W.-g. Extracellular ATP enhances in vitro invasion of prostate cancer cells by activating Rho GTPase and upregulating MMPs expression. *Cancer Letters* **293**, 189–197 (July 2010) (cit. on p. 85).
224. Chen, L., He, H.-y., Li, H.-m., Zheng, J., Heng, W.-j., You, J.-f. & Fang, W.-g. ERK1/2 and p38 pathways are required for P2Y receptor-mediated

- prostate cancer invasion. *Cancer Letters* **215**, 239–247 (Nov. 2004) (cit. on p. [85](#)).
225. Li, W.-H., Qiu, Y., Zhang, H.-Q., Liu, Y., You, J.-F., Tian, X.-X. & Fang, W.-G. P2Y2 receptor promotes cell invasion and metastasis in prostate cancer cells. *British Journal of Cancer* **109**, 1666–1675 (Sept. 2013) (cit. on p. [85](#)).
226. Shabbir, M., Thompson, C., Jarmulowicz, M., Mikhailidis, D. & Burnstock, G. Effect of extracellular ATP on the growth of hormone-refractory prostate cancer in vivo. *BJU international* **102**, 108–112 (July 2008) (cit. on p. [85](#)).
227. Orriss, I. R., Key, M. L., Hajjawi, M. O. R. & Arnett, T. R. Extracellular ATP Released by Osteoblasts Is A Key Local Inhibitor of Bone Mineralisation. *PLoS ONE* **8**, e69057 (July 2013) (cit. on p. [97](#)).
228. Shabbir, M., Ryten, M., Thompson, C., Mikhailidis, D. & Burnstock, G. Characterization of calcium-independent purinergic receptor-mediated apoptosis in hormone-refractory prostate cancer. *BJU International* **101**, 352–359 (Feb. 2008) (cit. on pp. [99](#), [132](#)).
229. Shabbir, M., Thompson, C., Jarmulowicz, M., Mikhailidis, D. & Burnstock, G. Effect of extracellular ATP on the growth of hormone-refractory prostate cancer in vivo. *BJU international* **102**, 108–112 (July 2008) (cit. on pp. [99](#), [132](#)).
230. Schnurr, M., Then, F., Galambos, P., Scholz, C., Siegmund, B., Endres, S. & Eigler, A. Extracellular ATP and TNF-alpha synergize in the activation and maturation of human dendritic cells. *Journal of Immunology* **165**, 4704–4709 (Oct. 2000) (cit. on p. [100](#)).
231. Kucher, B. M. & Neary, J. T. Bi-functional effects of ATP/P2 receptor activation on tumor necrosis factor-alpha release in lipopolysaccharide-stimulated astrocytes. *Journal of Neurochemistry* **92**, 525–535 (Feb. 2005) (cit. on p. [100](#)).
232. Lambert, C., Ase, A. R., Séguéla, P. & Antel, J. P. Distinct migratory and cytokine responses of human microglia and macrophages to ATP. *Brain, Behavior, and Immunity* **24**, 1241–1248 (Nov. 2010) (cit. on p. [100](#)).
233. Schulze-Lohoff, E., Hugo, C., Rost, S., Arnold, S., Gruber, A., Brüne, B. & Sterzel, R. B. Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors. *American Journal of Physiology* **275**, F962–F971 (Dec. 1998) (cit. on p. [100](#)).

-
234. Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. & Tewari, M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciences* **105**, 10513–10518 (July 2008) (cit. on p. 104).
235. Boll, K., Reiche, K., Kasack, K., Mörbt, N., Kretzschmar, A. K., Tomm, J. M., Verhaegh, G., Schalken, J., Bergen, M. v., Horn, F. & Hackermüller, J. MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma. *Oncogene* (Mar. 2012) (cit. on p. 105).
236. Viticchiè, G., Lena, A. M., Latina, A., Formosa, A., Gregersen, L. H., Lund, A. H., Bernardini, S., Mauriello, A., Miano, R., Spagnoli, L. G., Knight, R. A., Candi, E. & Melino, G. MiR-203 controls proliferation, migration and invasive potential of prostate cancer cell lines. *Cell Cycle* **10**, 1121–1131 (Apr. 2011) (cit. on p. 105).
237. Yi, R., Poy, M. N., Stoffel, M. & Fuchs, E. A skin microRNA promotes differentiation by repressing 'stemness'. *Nature* **452**, 225–229 (Mar. 2008) (cit. on p. 105).
238. Lena, A. M., Shalom-Feuerstein, R., Rivetti di Val Cervo, P., Aberdam, D., Knight, R. A., Melino, G. & Candi, E. miR-203 represses 'stemness' by repressing DeltaNp63. *Cell Death and Differentiation* **15**, 1187–1195 (July 2008) (cit. on p. 105).
239. Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., Hausen, A. z., Brunton, V. G., Morton, J., Sansom, O., Schüller, J., Stemmler, M. P., Herzberger, C., Hopt, U., Keck, T., Brabletz, S. & Brabletz, T. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nature Cell Biology* **11**, 1487–1495 (Nov. 2009) (cit. on p. 105).
240. Storey, J. D. A direct approach to false discovery rates. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)* **64**, 479–498 (Aug. 2002) (cit. on pp. 111, 112).
241. Birmingham, A., Selfors, L. M., Forster, T., Wrobel, D., Kennedy, C. J., Shanks, E., Santoyo-Lopez, J., Dunican, D. J., Long, A., Kelleher, D., Smith, Q., Beijersbergen, R. L., Ghazal, P. & Shamu, C. E. Statistical Methods for

- Analysis of High-Throughput RNA Interference Screens. *Nature methods* **6**, 569–575 (Aug. 2009) (cit. on p. 118).
242. Place, R. F., Li, L.-C., Pookot, D., Noonan, E. J. & Dahiya, R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proceedings of the National Academy of Sciences* **105**, 1608–1613 (Feb. 2008) (cit. on p. 125).
243. Hofmann, M.-C., Jeltsch, W., Brecher, J. & Walt, H. Alkaline Phosphatase Isozymes in Human Testicular Germ Cell Tumors, Their Precancerous Stage, and Three Related Cell Lines. *Cancer Research* **49**, 4696–4700 (Sept. 1989) (cit. on p. 128).
244. Armstrong, A. J., Bitting, R. L., Kemeny, G. & George, D. J. Evidence for circulating tumor cell (CTC) alkaline phosphatase (AP) expression in men with bone-metastatic castration-resistant prostate cancer (CRPC) during abiraterone acetate treatment. *Journal of Clinical Oncology* **32** (2014) (cit. on p. 129).
245. Liu, J., Nam, H. K., Campbell, C., Gasque, K. C. d. S., Millán, J. L. & Hatch, N. E. Tissue-nonspecific alkaline phosphatase deficiency causes abnormal craniofacial bone development in the *Alpl*^{-/-} mouse model of infantile hypophosphatasia. *Bone* **67**, 81–94 (Oct. 2014) (cit. on p. 129).
246. Narisawa, S., Fröhlander, N. & Millán, J. L. Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Developmental Dynamics* **208**, 432–446 (Mar. 1997) (cit. on pp. 129, 132).
247. Ochiai-Shino, H., Kato, H., Sawada, T., Onodera, S., Saito, A., Takato, T., Shibahara, T., Muramatsu, T. & Azuma, T. A Novel Strategy for Enrichment and Isolation of Osteoprogenitor Cells from Induced Pluripotent Stem Cells Based on Surface Marker Combination. *PLoS ONE* **9**, e99534 (June 2014) (cit. on p. 129).
248. Tilgner, K., Atkinson, S. P., Golebiewska, A., Stojković, M., Lako, M. & Armstrong, L. Isolation of Primordial Germ Cells from Differentiating Human Embryonic Stem Cells. *Stem Cells* **26**, 3075–3085 (Dec. 2008) (cit. on p. 129).
249. Liu, K.-C., Yo, Y.-T., Huang, R.-L., Wang, Y.-C., Liao, Y.-P., Huang, T.-S., Chao, T.-K., Lin, C.-K., Weng, S.-J., Ma, K.-H., Chang, C.-C., Yu, M.-H. & Lai, H.-C. Ovarian cancer stem-like cells with induced translineage-

- differentiation capacity and are suppressed by alkaline phosphatase inhibitor. *Oncotarget* **4**, 2366–2382 (Nov. 2013) (cit. on p. [129](#)).
250. Anagnostou, F., Plas, C. & Forest, N. Ecto-alkaline phosphatase considered as levamisole-sensitive phosphohydrolase at physiological pH range during mineralization in cultured fetal calvaria cells. *Journal of Cellular Biochemistry* **60**, 484–494 (Mar. 1996) (cit. on p. [130](#)).
 251. Uhlén, M., Björling, E., Agaton, C., Szigartyo, C. A.-K., Amini, B., Andersen, E., Andersson, A.-C., Angelidou, P., Asplund, A., Asplund, C., Berglund, L., Bergström, K., Brumer, H., Cerjan, D., Ekström, M., Elobeid, A., Eriksson, C., Fagerberg, L., Falk, R., Fall, J., Forsberg, M., Björklund, M. G., Gumbel, K., Halimi, A., Hallin, I., Hamsten, C., Hansson, M., Hedhammar, M., Hercules, G., Kampf, C., Larsson, K., Lindskog, M., Lodewyckx, W., Lund, J., Lundeborg, J., Magnusson, K., Malm, E., Nilsson, P., Odling, J., Oksvold, P., Olsson, I., Oster, E., Ottosson, J., Paavilainen, L., Persson, A., Rimini, R., Rockberg, J., Runeson, M., Sivertsson, A., Sköllermo, A., Steen, J., Stenvall, M., Sterky, F., Strömberg, S., Sundberg, M., Tegel, H., Tourle, S., Wahlund, E., Waldén, A., Wan, J., Wernérus, H., Westberg, J., Wester, K., Wrethagen, U., Xu, L. L., Hober, S. & Pontén, F. A human protein atlas for normal and cancer tissues based on antibody proteomics. *Molecular & cellular proteomics* **4**, 1920–1932 (Dec. 2005) (cit. on p. [130](#)).
 252. Whyte, M. P., Greenberg, C. R., Salman, N. J., Bober, M. B., McAlister, W. H., Wenkert, D., Van Sickle, B. J., Simmons, J. H., Edgar, T. S., Bauer, M. L., Hamdan, M. A., Bishop, N., Lutz, R. E., McGinn, M., Craig, S., Moore, J. N., Taylor, J. W., Cleveland, R. H., Cranley, W. R., Lim, R., Thacher, T. D., Mayhew, J. E., Downs, M., Millán, J. L., Skrinar, A. M., Crine, P. & Landy, H. Enzyme-Replacement Therapy in Life-Threatening Hypophosphatasia. *New England Journal of Medicine* **366**, 904–913 (Mar. 2012) (cit. on p. [131](#)).
 253. Hsu, S.-D., Tseng, Y.-T., Shrestha, S., Lin, Y.-L., Khaleel, A., Chou, C.-H., Chu, C.-F., Huang, H.-Y., Lin, C.-M., Ho, S.-Y., Jian, T.-Y., Lin, F.-M., Chang, T.-H., Weng, S.-L., Liao, K.-W., Liao, I.-E., Liu, C.-C. & Huang, H.-D. miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. *Nucleic Acids Research* **42**, D78–D85 (Jan. 2014) (cit. on p. [131](#)).
 254. Zhang, L., Wang, X. & Chen, P. MiR-204 down regulates SIRT1 and reverts SIRT1-induced epithelial-mesenchymal transition, anoikis resistance and in-

- vasion in gastric cancer cells. *BMC Cancer* **13**, 290 (June 2013) (cit. on p. 131).
255. Qiu, Y.-h., Wei, Y.-p., Shen, N.-j., Wang, Z.-c., Kan, T., Yu, W.-l., Yi, B. & Zhang, Y.-j. miR-204 inhibits epithelial to mesenchymal transition by targeting slug in intrahepatic cholangiocarcinoma cells. *Cellular Physiology and Biochemistry* **32**, 1331–1341 (2013) (cit. on p. 131).
 256. Huang, J., Zhao, L., Xing, L. & Chen, D. MicroRNA-204 Regulates Runx2 Protein Expression and Mesenchymal Progenitor Cell Differentiation. *Stem cells* **28**, 357–364 (Feb. 2010) (cit. on p. 131).
 257. Chen, Q., Liu, W., Sinha, K. M., Yasuda, H. & de Crombrughe, B. Identification and Characterization of MicroRNAs Controlled by the Osteoblast-Specific Transcription Factor Osterix. *PLoS ONE* **8**, e58104 (Mar. 2013) (cit. on p. 131).
 258. Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W. J. & Pandolfi, P. P. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* **465**, 1033–1038 (June 2010) (cit. on p. 131).
 259. MacGregor, G. R., Zambrowicz, B. P. & Soriano, P. Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. *Development* **121**, 1487–1496 (May 1995) (cit. on p. 132).
 260. Li, W.-H., Qiu, Y., Zhang, H.-Q., Liu, Y., You, J.-F., Tian, X.-X. & Fang, W.-G. P2Y2 receptor promotes cell invasion and metastasis in prostate cancer cells. *British Journal of Cancer* **109**, 1666–1675 (Sept. 2013) (cit. on p. 132).
 261. Yang, Q., Du, J. & Zu, L. Overexpression of CD73 in prostate cancer is associated with lymph node metastasis. *Pathology oncology research* **19**, 811–814 (Oct. 2013) (cit. on p. 132).
 262. Jia, Z., Wang, Y., Sawyers, A., Yao, H., Rahmatpanah, F., Xia, X.-Q., Xu, Q., Pio, R., Turan, T., Koziol, J. A., Goodison, S., Carpenter, P., Wang-Rodriguez, J., Simoneau, A., Meyskens, F., Sutton, M., Lernhardt, W., Beach, T., Monforte, J., McClelland, M. & Mercola, D. Diagnosis of Prostate Cancer Using Differentially Expressed Genes in Stroma. *Cancer Research* **71**, 2476–2487 (Apr. 2011) (cit. on p. 132).
 263. Feng, L., Sun, X., Csizmadia, E., Han, L., Bian, S., Murakami, T., Wang, X., Robson, S. C. & Wu, Y. Vascular CD39/ENTPD1 directly promotes tumor

- cell growth by scavenging extracellular adenosine triphosphate. *Neoplasia* **13**, 206–216 (Mar. 2011) (cit. on p. [132](#)).
264. Zimmermann, H. Prostatic acid phosphatase, a neglected ectonucleotidase. *Purinergic Signalling* **5**, 273–275 (Sept. 2009) (cit. on p. [132](#)).
265. Zylka, M. J., Sowa, N. A., Taylor-Blake, B., Twomey, M. A., Herrala, A., Voikar, V. & Vihko, P. Prostatic Acid Phosphatase Is an Ectonucleotidase and Suppresses Pain by Generating Adenosine. *Neuron* **60**, 111–122 (Oct. 2008) (cit. on p. [132](#)).
266. Schoolmeesters, A., Eklund, T., Leake, D., Vermeulen, A., Smith, Q., Force Aldred, S. & Fedorov, Y. Functional Profiling Reveals Critical Role for miRNA in Differentiation of Human Mesenchymal Stem Cells. *PLoS ONE* **4**, e5605 (May 2009) (cit. on p. [133](#)).
267. Zhou, L., Qi, X., Potashkin, J. A., Abdul-Karim, F. W. & Gorodeski, G. I. MicroRNAs miR-186 and miR-150 down-regulate expression of the pro-apoptotic purinergic P2X7 receptor by activation of instability sites at the 3'-untranslated region of the gene that decrease steady-state levels of the transcript. *The Journal of Biological Chemistry* **283**, 28274–28286 (Oct. 2008) (cit. on p. [133](#)).
268. Huang, S., Chen, Y., Wu, W., Ouyang, N., Chen, J., Li, H., Liu, X., Su, F., Lin, L. & Yao, Y. miR-150 promotes human breast cancer growth and malignant behavior by targeting the pro-apoptotic purinergic P2X7 receptor. *PLoS ONE* **8**, e80707 (2013) (cit. on p. [133](#)).