Altered lysosomal biogenesis in prostate cancer

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List of Abbreviations

AKT	RAC-alpha serine/threonine-protein kinase
AMACR	α-Methylacyl-CoA racemase
Appl1	DCC-interacting protein 13-alpha
Appl2	DCC-interacting protein 13-beta
AR	Androgen receptor
ARE	Androgen response element
BCR	Biochemical recurrance
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CA 15-3	Carbohydrate antigen marker 15-3
CA 19-9	Carbohydrate antigen marker 19-9
cDNA	Complementary deoxyribonucleic acid
CEA	Carcinoma embryonic antigen
CRP	C-reactive protein
DEPC	Diethylpyrocarbonate
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
DOC	Sodium deoxycholate
DPBS	Dulbecco's PBS
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early Endosome Antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated protein kinase
ESCRT	Endosome sorting complex required for transport
FCS	Foetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
hK 1-15	Human kallikrein 1-15
HPV	Human papilloma virus
HRP	Horseradish peroxidase
IGF	Insulin growth factor
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte growth factor
KLK 1-15	Kallikrein 1-15
LAMP	Lysosome associated membrane protein
LIMP	Lysosome integral membrane protein
MALDI	Matrix-assisted laser desorption/ionisation
MAPK	Mitogen activated protein kinases
MVB	Multivesicular body
PAP	Prostatic acid phosphatase
PCA3	Prostate cancer antigen 3
PCR	Polymerase chain reaction
pH	Logarithm of the reciprocal of hydrogen ion concentration
PI3P	Phosphatidylinositol 3-phosphate
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time PCR
Rab	Ras superfamily of monomeric G proteins
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SE	Standard error
Shh	Sonic hedgehog
SV40	Simian vacuolating virus 40
TBS	Tris-buffered saline
TfR	Transferrin receptor
β-CG	Beta-glucocerebrosidase
β-hCG	β-human chorionic gonadotrophin

Abstract

Prostate cancer is the most common form of cancer in males, affecting approximately one in five men by the time they reach the age of 85. In Australia, more than 19,000 men are diagnosed with prostate cancer and 3,000 die from this disease. Current diagnostic tests for prostate cancer are limited in both sensitivity and accuracy, and are unable to stratify patients at risk of progressive disease and mortality; therefore, there is a need for a sensitive and specific prostate cancer test to implement early and appropriate therapy.

Lysosomal enzymes have previously been implicated in cancer processes, and a number of these proteins have been evaluated in the diagnosis and prognosis of cancers and for the assessment of metastatic potential. This suggested that lysosomal biogenesis may be altered in prostate cancer, and that lysosomal related proteins might provide a novel source of markers to facilitate early detection and prognosis of prostate cancer. Endosomes and lysosomes are directly involved in the critical processes of energy metabolism, cell division, intracellular signalling and cellular secretion, and therefore have a direct role in cancer pathogenesis. Despite the potential association between lysosomes and disease pathogenesis, there has not been a systematic study of endosome-lysosome biogenesis in prostate cancer.

Cell lines have been utilised heavily for prostate cancer biomarker discovery; however, there have been significant problems with these studies, in terms of the number and type of prostate cell lines examined. Studies involving multiple non-malignant control cell lines have been limited, despite the number of prostate cell lines available, and this may have been detrimental to effective biomarker discovery. Protein and gene expression data for

the common markers of prostate specific antigen, androgen receptor and kallikrein 2 has previously been compiled, although a systematic study to identify novel biomarkers using multiple non-malignant and prostate cancer cell lines has not been performed. In this study, three non-malignant and four prostate cancer cell lines were selected for the characterisation of lysosomal protein and gene expression. This analysis revealed phenotypes in some cell lines that may not be representative of prostate cancer and may be artefacts of their *in vitro* immortalisation and culture.

The differential secretion of endosomal proteins, but not lysosomal proteins, from prostate cancer cells prompted further investigation of the endosome compartment and endosome biogenesis. This exploration revealed elevated gene and protein expression of critical machinery components that are required for endosome biogenesis and endocytosis. Moreover, the endosome compartments were spatiotemporally affected in prostate cancer cells and this may affect the signalling pathways within the cell and proliferative and apoptotic processes.

Meta-analysis of multiple cDNA microarray databases of prostate cancer and non-malignant prostate tissue established the validity of the *in vitro* derived data and suggested that lysosomal and endosomal biogenesis may be altered during cancer progression, providing a valuable tool for prognosis. Furthermore, a panel of three endosomal genes provided a mode of stratifying patients at risk from cancer recurrence, particularly in those patients with low blood serum concentrations of prostate specific antigen. It was concluded that endosome-lysosome biogenesis is significantly altered in prostate cancer cells. These novel observations will lead to the development of new biomarkers that aid in the diagnosis and prognosis of prostate cancer.

Declaration

I declare that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university. To the best of my knowledge it does not contain any material previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.



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Patent

Australia Patent Application No. 2013902141, "Method for detecting prostate cancer", Provisional (filing date 13th June 2013) (Brooks, DA)

Conference presentations

Johnson IRD, Parkinson-Lawrence E, Hopwood JJ, Brooks DA. Altered late endosome biogenesis and secretion in prostate cancer. Australian Society of Medical Research SA Branch Annual Meeting, Adelaide. June 2011

Johnson IRD, Parkinson-Lawrence E, Shandala T, Brooks DA. Altered endosome biogenesis in prostate cancer. Australian Society of Medical Research SA Branch Annual Meeting, Adelaide. June 2012

Johnson IRD, Parkinson-Lawrence E, Butler L, Shandala T, Brooks DA. Altered endosome biogenesis in prostate cancer. Combio 2012, Adelaide. October 2012

Chapter 1

Introduction

1.1 The prostate

Prostate cancer is the most common form of cancer in males from developed countries. In 2009, more than nineteen thousand Australian men were diagnosed with prostate cancer, and there were three thousand deaths, making this disease one of the largest causes of cancer-related deaths in men. The prostate-specific antigen assay is currently used for prostate cancer diagnosis; however, this assay suffers from a high percentage of false-positive results and does not stratify patients by risk. Early detection of prostate cancer is critical as it enables effective treatment and reduced mortality. Understanding the cell biology of prostate cancer is important in order to develop new approaches for the early diagnosis, prognosis and treatment of prostate cancer. This thesis aimed to investigate altered lysosome and endosome biology in prostate cancer, which may provide a new avenue for the investigation of more accurate and specific biomarkers for prostate cancer diagnosis and prognosis.

1.1.1 Structure and function of the prostate

The prostate gland is located in the subperitoneal compartment between the pelvic diaphragm and the peritoneal cavity and surrounds the proximal urethra exiting the bladder (Figure 1.1). The gland is comprised of three distinct regions or zones, characterised by their functions and anatomic landmarks (Figure 1.2). Branching glands and ducts comprise the peripheral, central and transition zones and are lined with secretory epithelial and basal cells (Isaacs 1983; McNeal 1988). A fourth anterior zone is composed of fibromuscular stroma that is capable of restricting urinary flow through the urethra. Though the peripheral zone and transition zone originate from the same embryonic tissue, the transition zone is composed of fibromuscular tissue and the

peripheral zone is composed of a connective framework of stromal cells, fibroblasts and smooth muscle cells (Fine & Reuter 2012).

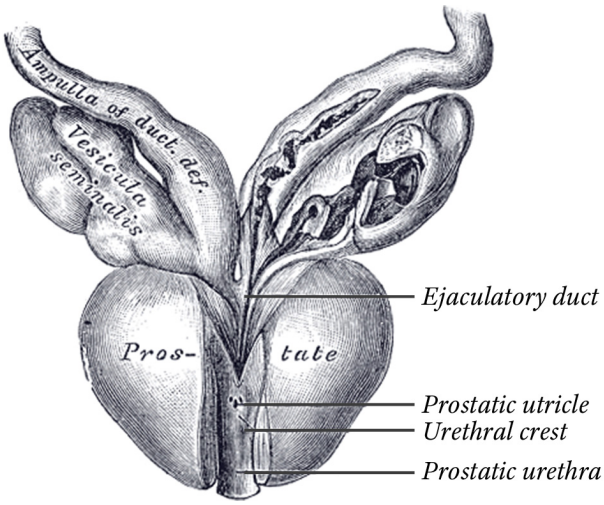


Figure 1.1 – Anatomy of the prostatic region (adapted from Gray's Anatomy of the Human Body, 1918).

The glandular tissue of the prostate secretes prostate specific antigen (PSA), prostatic acid phosphatase (PAP), citric acid, zinc and polyamines that aid the function of spermatozoa (Gillenwater 2002; Isaacs 1983; Janne, Alhonen & Leinonen 1991). Distinct cell types within the prostate secrete variable amounts of these components and express receptors that influence cell proliferation and gland development. Epithelial cells secrete PSA and PAP and express androgen receptor (AR); basal cells express epidermal growth factor receptors (EGFR) and do not express or secrete PSA or PAP protein (Maygarden, Strom & Ware 1992); fibroblastic cells, stimulated by androgen, secrete epidermal and insulin growth factors (EGF and IGF) that may induce cell proliferation via paracrine activation. Thus, regulation of prostate cells is critical for the correct development and maintenance of the gland upon its maturation.

The development, maintenance and function of prostate tissue is regulated by androgens (steroid hormones). Secretory epithelial cells expressing androgen receptors require continuous stimulation by androgen to maintain viability. A disturbance in the androgen supply or function of the androgen receptor will result in either: apoptosis; atrophy and glandular involution (Montalvo et al. 2000; Sandford, Searle & Kerr 1984); or impeded growth and function (Simanainen et al. 2007). In the normal development and function of the prostate, the enzyme 5α-reductase converts the androgen testosterone to dihydrotestosterone (DHT) and this steroid subsequently binds and induces a conformational change of the androgen receptor, exposing a DNA binding site (Marks 2004). The ligand-bound androgen receptor initiates a signalling cascade, stimulating its trafficking into the nucleus and subsequent binding of androgen response elements in the promoter region of target genes (Jasuja et al. 2009). Target genes may be involved in the proliferation, differentiation or apoptosis of cells (Ware 1993), thus these functions can be regulated through modulation of androgen supply and androgen receptor function.

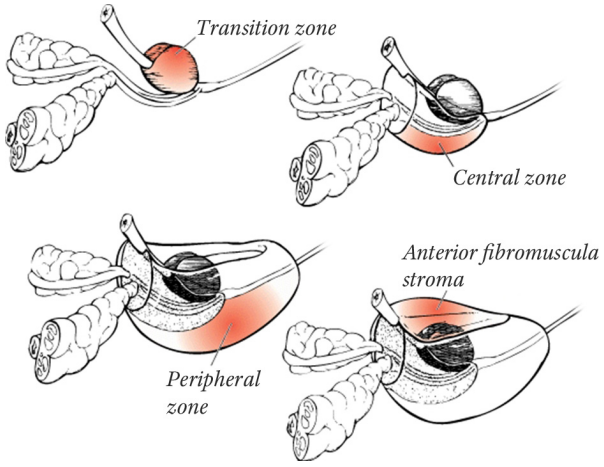


Figure 1.2 – Zones of the prostate (adapted from Hong, WK et al. 2010).

1.2 Diseases of the prostate

The prostate frequently sustains a variety of benign lesions such as prostatic atrophy and benign prostatic hyperplasia, basal cell hyperplasia, adenosis and sclerosing adenosis (Harik & O'Toole 2012); in addition to prostatic intraepithelial neoplasia (PIN) and prostate cancer (PCa) malignancies.

1.2.1 Benign prostatic hyperplasia

Benign prostatic hyperplasia is the most common disease of the prostate and develops from stromal and glandular alterations (Timms & Hofkamp 2011). Nodules that alter epithelial cell differentiation and stimulate growth (McNeal 1988) form within the transition zone, and to a lesser extent at the boundary of the peripheral and transition zone (Harik & O'Toole 2012). Benign prostatic hyperplasia and the associated pathologies of lower urinary tract symptoms and bladder-outlet obstruction affect a large proportion of men (depicted in Figure 1.3) and are mediated by both testosterone and DHT that are required for normal prostate development (Hannema & Hughes 2007). Indeed, reduction in prostate volume and lower urinary tract symptoms is achieved with inhibitors to 5α-reductase, which normally converts testosterone to DHT (Robert, Descazeaud & de la Taille 2011). Advanced benign prostatic hyperplasia may result in basal cell hyperplasia, where basal cells of ducts and acini become rounded, multi-layered and surrounded by secretory cells that express prostate specific antigen and prostatic acid phosphatase (McNeal 1988). Whilst benign prostatic hyperplasia is distinguishable from prostate cancer histologically, there are significant biochemical similarities with prostate cancer. Prostate specific antigen displays increased expression and secretion in benign prostatic hyperplasia and is further elevated simultaneously with age (Bo et al. 2003). Thus, the PSA assay is

unable to adequately distinguish between benign or cancer tissue in patients, resulting in false-positive diagnoses and unnecessary and invasive therapy.

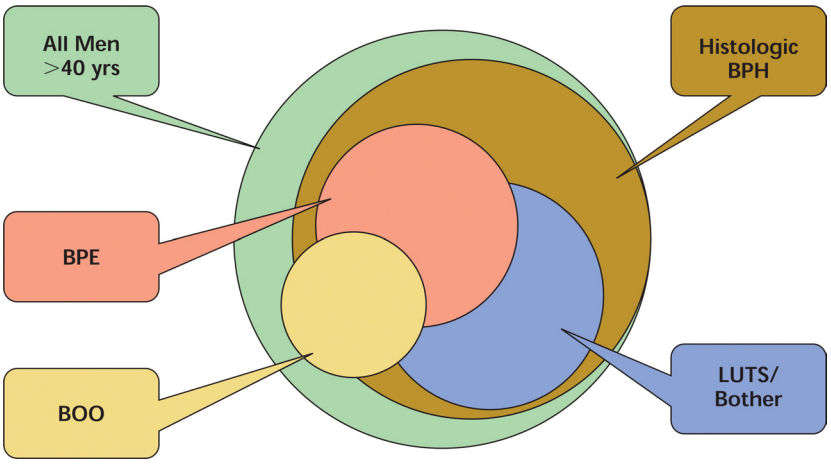


Figure 1.3 – Representation of the proportion of men with benign prostatic diseases (adapted from Roehrborn 2005).

BPH: Benign prostatic hyperplasia; BPE: Benign prostatic enlargement;

BOO: Associated bladder outlet obstruction; LUTS: Lower urinary tract symptoms.

1.2.2 Prostatic intraepithelial neoplasia

Prostatic intraepithelial neoplasia (PIN) is a pre-cancerous lesion of the prostate, characterised by altered gland architecture. These lesions form from altered proliferation of ductal and acini-derived cells, and altered location of epithelial cells that oscillate from a basal to lumen localisation (Brawer et al. 1985). The cells of PIN tissue display nucleolar enlargement in a similar manner to prostate cancer (Bostwick et al. 2004). Prostatic intraepithelial neoplasia lesions are multicentric, occurring in 72 % of radical prostatectomies and located in either transition zone (7 %) or non-transition zone areas, such as the peripheral zone (63 %) or a combination of all zones (2 %). Prostatic intraepithelial neoplasia lesions are classed on a 3-point scale as either low (1) or high-grade (2-3), the latter being the more commonly observed pathology. Alterations in gland

architecture coincide with a PIN volume that increases simultaneously with pathological grade, Gleason grade and invasion potential, similar to that of primary prostate cancer (Qian, Wollan & Bostwick 1997). Prostatic intraepithelial neoplasia tissue exhibits frequent reductions in telomere length and frequent loss, gain or translocation of chromosomes (Bostwick & Cheng 2012). These alterations are also characteristic of primary prostate cancer and genesis of PIN tissue provides preliminary evidence of prostate cancer onset. Indeed, high grade PIN is frequently found alongside prostate cancer compared to benign prostatic hyperplasia (Bostwick & Cheng 2012). The transcription factor C-myc is upregulated in PIN and primary cancer, significantly elevated compared to benign tissue, and may be used in PIN diagnosis using an immunological assay on biopsy samples (Bostwick & Cheng 2012). Despite the alterations in biology and marker expression in PIN, there remains a high rate of false-positive detection. Benign prostatic hyperplasia or low-grade PIN may be classed as high-grade PIN, and this may result in unnecessary biopsies and PSA testing in active surveillance. There is a critical need for markers that can adequately distinguish between benign conditions and stages of PIN that are most likely to progress to an invasive cancer phenotype.

1.2.3 Prostate cancer

1.2.3.1 Epidemiology

One in five men in Australia will be diagnosed with prostate cancer by the age of 85; each year, more than nineteen thousand Australian men are diagnosed with, and three thousand die directly from prostate cancer (AIHW 2012). The risk of prostate cancer onset is highly correlated with advancing age; the majority of symptomatic incidences occur in men over 60 years and rarely in men younger than 45 years of age. Mortality resulting from prostate

cancer follows a similar trend, with increasing mortality rate associated with age progression (Figure 1.4).

Autopsies of men aged less than 50 years who have died from independent causes showed a proportion of men with prostate cancer foci development (Sandhu & Andriole 2012). Approximately 10 % of men below the age of 30 had prostate cancer, increasing to more than 70 % in men age 60-79 (Sandhu & Andriole 2012). The risk of mortality from prostate cancer is approximately 3 %, despite a 20 % diagnosis rate (Siegel et al. 2011). The inability to distinguish between patients with clinically relevant cancer and patients with less severe forms of the disease result in significant over-diagnosis. Consequently, there is a significant burden placed on patients and healthcare providers as a result of unnecessary and costly intervention.

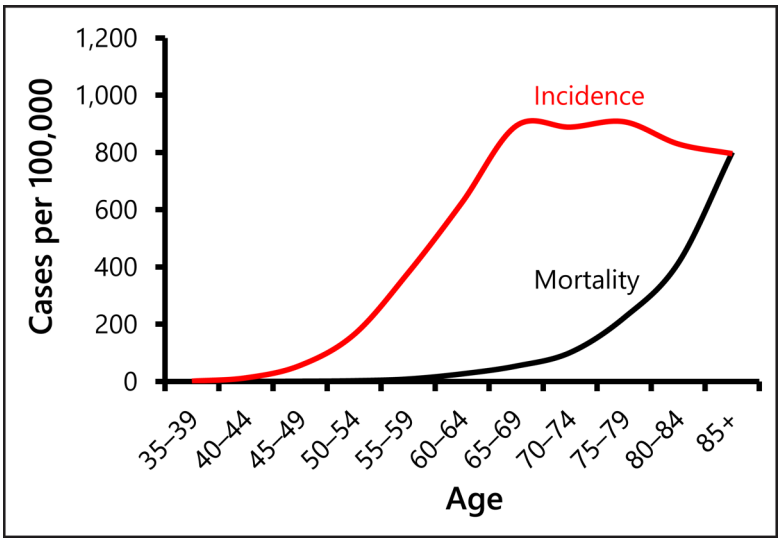


Figure 1.4 – Prostate cancer incidence (2009) and mortality (2007) in Australia[1].

[1] aihw.gov.au/acim_books/ (accessed July 2013)

1.2.3.2 Detection strategies for prostate cancer

A physical examination of the prostate by digital rectal examination and the quantitation of the prostate specific antigen (PSA) concentration in blood serum can be performed on men presenting with lower urinary tract symptoms, or during routine testing. The digital rectal examination is a conventional technique used by physicians to detect palpable changes and asymmetry to the surface of the prostate. The inability to palpitate the entire prostate gland and detection of only advanced-stage local cancers limits its diagnostic effectiveness (Ilic et al. 2007). The prostate specific antigen protein, kallikrein 3 (hK3), is significantly upregulated upon androgen stimulation, when compared to non-stimulated cells (Clegg et al. 2002). Kallikrein 3 is produced by prostate luminal epithelial cells and is the most prominent secretory protein produced by the prostate. The altered morphology of secretory epithelial cells and prostate gland that occurs upon onset of prostate cancer results in the release of hK3 into the blood serum (Kuriyama et al. 1980). Whilst the concentration of hK3 in seminal plasma varies between 390 and 3000 ng/mL (Wang, TJ et al. 1998), in normal physiology blood serum levels range between 0–2.5 ng/mL in men aged 40-49 years to between 0-6.5 ng/mL in men aged over 70 years (Oesterling et al. 1993). Concentrations of hK3 that are greater than 4 ng/mL are suggested to be abnormal, and prompts further investigation through histological analysis of prostate biopsies. The disease stage is measured from these biopsy samples and given a Gleason grade and staging-grade based on visualisation of altered cell differentiation and altered architectural patterns within the gland. The scoring and grading of tissue is used for the prognosis of the disease (Sogani et al. 1985). This is further combined with hK3 serum concentration to stratify patients by risk and guide appropriate therapy. Known variables such as T-stage, Gleason score, PSA and histology results from outcome data on large cohorts of prostate

cancer patients has been used to develop Nomograms, which is a statistically derived tool that estimates the risk of metastatic spread, lymph node involvement or recurrence following treatment (Kattan et al. 1997). Nomograms are more accurate at predicting mortality than measurements of PSA alone (Teeter et al. 2013), and the incorporation of additional markers may improve clinical treatment decisions.

The digital rectal examination has poor predictive value for men with low hK3 blood-serum concentrations (Schroder, FH et al. 1998), and hK3 does not discriminate between cancer or conditions such as BPH (Bunting 1995; De Nunzio et al. 2011; Nadler et al. 1995). Moreover, hK3 does not distinguish between aggressive or more slow-growing cancer, resulting in significant over-diagnosis (Schroder, FH et al. 2009). Only 25 % of men with an abnormal concentration of hK3 have a detectable cancer by prostate biopsy (Barry 2001). Furthermore, elevated hK3 is not present in all prostate cancers, often because of gene deletions or splice variants that alter gene and protein expression in some prostate cancer patients (Rodriguez et al. 2013), producing many false-negative results. The concentration of hK3 in blood is highly variable and diagnosis using this marker is therefore often inaccurate. Consequently, the PSA assay has been adapted to detect alternate forms or variations in hK3 expression to improve the reliability and value of prostate cancer diagnosis. These methods include measuring hK3 over a period of time (PSA velocity), determining levels of free and protein-bound hK3, or calculating a patient's hK3 density from serum concentration and prostate volume (Cazares et al. 2010; Hoffman 2011). Despite these developments of the PSA assay, the predictive accuracy of PSA remains limited and does not adequately improve diagnosis or early detection of prostate cancer (Vickers 2013). Moreover, prostate cancer screening is not advised since the detriment to patients resulting from false-negative or false-positive diagnoses outweigh the potential

benefits (The Cancer Council Australia, May 2010; Moyer 2012; Vogelzang et al. 2012). There is therefore an urgent need for a more specific and accurate method for prostate cancer diagnosis that will allow early detection and the selection of appropriate patients for intervention therapy and discrimination between disease grades.

1.2.3.3 Additional prostate cancer markers

Other proteins have been associated with prostate cancer and the increased expression of these markers have been evaluated for their prognostic capabilities (Table 1.1). However, these additional markers are not specific to primary stages of prostate cancer disease, thus serve limited diagnostic potential. The amount of β-human chorionic gonadotrophin (β-hCG) was increased in the serum and urine of patients with prostate cancer, and was associated with low-grade, but not advanced high-grade or metastatic disease (Daja et al. 2000). Interestingly, expression of β-hCG has been found to promote prostate cancer cell migration and invasion (Wu & Walker 2006). Despite these associations with cancer, β-hCG detection in serum or urine does not predict a significant outcome for prostate cancer patients (Otite et al. 2006). The carbohydrate antigen marker 15-3 (CA 15-3) is indicative of various types of tumours and although this marker is not unique to prostate cancer, CA 15-3 may be used to aid in diagnosis when prostate specific antigen results are not clear (Falsaperla et al. 2003). The carbohydrate antigen marker 19-9 (CA 19-9) protein is present in biopsy and autopsy samples from prostate cancer patients within primary and metastatic sites, however it has only been proven to have minimal diagnostic value for prostate cancer (Sella et al. 2000).

Marker	Source or type of marker	Malignancy association	Additional associations	Benign pathologies	Approximate sensitivity for principal cancer
β-hCG	Trophoblastic tissue	NSGCT, gestational trophoblastic	Gastrointestinal, bladder, **prostate**	Pregnancy, gestational trophoblastic disease, hypogonadism	NSGCT: 20 % of early stage, 85 % late stage
CA 15-3	Monoclonal antibody to MUC1 glycoprotein (on the apical surface of epithelial cells)	Breast	Ovary, lung, HCC, **prostate**	Chronic hepatitis, hepatic cirrhosis, sarcoidosis, tuberculosis, systemic lupus erythematosus	20-40 % (early stage)
CA 19-9	Intracellular adhesion molecule	Pancreatic, biliary tree	Colorectal, oesophageal, HCC, gastric, **prostate**	Pancreatitis, biliary disease, cirrhosis	80-90 % (pancreatic), 60-70 % (biliary tract). Predictive value <1 % in asymptomatic patients of pancreatic cancer, >97 % with symptoms
CRP	Homopentamer secreted by epithelial cells	**Prostate**	Colorectal, Ovarian	Cardiovascular disease, inflammation from tissue injury/infection	<45 %
CEA	Oncofoetal glycoprotein expressed by normal mucosal cells	Colorectal	Breast, lung, gastric, pancreatic, thyroid (medullary), head and neck, cervical, hepatic, lymphoma, melanoma, **prostate**	Peptic ulcer disease, inflammatory bowel disease, pancreatitis, hypothyroidism, cirrhosis, biliary obstruction	<25 % (early stage), 75 % (late stage), 80 % (recurrence). Low organ specificity
PSA	Glycoprotein produced by prostatic epithelium	**Prostate**		Prostatitis, benign prostatic hyperplasia, prostatic trauma, post-ejaculation	>75 % (confined to organ)
PAP	Prostatic acid phosphatase	**Prostate**	Testicular, leukemia, non-Hodgkin's lymphoma		<75 %

Table 1.1 – Prostate cancer markers (adapted from Perkins et al. 2003).

C-reactive protein levels are found to be elevated in the circulatory system of prostate cancer patients (Kato et al. 2006) and this marker can reflect disease progression. Secretion of C-reactive protein into blood serum may further indicate benign inflammation and is not specific to prostate cancer; therefore, it may only be used to monitor progression late in tumour development. Moreover, C-reactive protein may be used to estimate overall survival upon recurrence of an androgen-independent form of prostate cancer following therapy (Ito et al. 2011). Carcinoma embryonic antigen is utilised as a secondary marker in prostate cancer biopsies, and is a potential marker of poor prognosis (Sella et al. 2000; Sturgeon et al. 2008). The specificity of these markers varies between 70 and 98 % whilst their sensitivity to prostate cancer is very low. The sensitivity of hK3 for prostate cancer varies in sensitivity from a maximum of 84 % in the first three years post-diagnosis, to just 30 % after 10 years (Gann, Hennekens & Stampfer 1995). Consequently, there is a critical need for more specific and sensitive markers to enable consistent diagnosis, prognosis and surveillance of the disease.

Kallikrein 3 is one of fifteen kallikrein family members (kallikrein 1-15), several of which are associated with cancer development (Diamandis & Yousef 2002). Analogous to elevated hK3 in prostate cancer, kallikrein 2 (hK2) serum concentration correlates markedly with prostate cancer development, particularly in cancers expressing lower amounts of hK3 (Alexander et al. 2001). In addition to protein secretion, the ratio of kallikrein 2 gene expression (*KLK2*) to kallikrein 3 gene expression (*KLK3*) also increases in advancing cancers, and may be able to distinguish aggressive disease (Lintula et al. 2005). A combined panel of markers is more accurate at predicting prostate cancer onset and disease stage than a single marker and the combination of hK2 and hK3 can provide more accurate diagnosis (Vickers et al. 2011). Prostate specific membrane antigen (PSMA)

is elevated in prostate cancer, however its prognostic capacity compared to PSA has not been clearly defined as the secretion of PSMA correlates with age, and is secreted from normal tissue and non-specific cancers (Kinoshita et al. 2006; Silver et al. 1997). Prostate cancer antigen 3 (*PCA3*) is a noncoding mRNA that shows elevated expression in prostate cancer and metastatic tissue and was detected in prostate epithelial cells that were released into the urinary tract and excreted in urine (Tinzl et al. 2004). There is insufficient evidence that *PCA3* improves intermediate to long-term health outcomes (Bradley et al. 2013) however, the diagnosis of cancer was improved when combined with PSA serum concentrations above 10 ng/mL (Neves et al. 2013).

1.2.3.4 Cancer development and progression

Whilst the risk of prostate cancer incidence increases with age, there are multiple pathways and factors implicated in the onset of prostate cancer resulting in disease heterogeneity. The androgen receptor pathway is altered in 56 % of primary prostate cancers and 100 % of metastatic prostate cancers (Taylor et al. 2010), suggesting that the androgen receptor is critical to the initiation and progression of prostate cancer (Hodgson, Bowden & Agoulnik 2012). Moreover, increased cell proliferation may be induced by interleukin-6 secretion (Azevedo et al. 2011; Salman et al. 2012) which causes an inflammatory response and increases androgen synthesis (Chun et al. 2009). The elevated expression of interleukins such as interleukin-6 occurs in aging men (Woods et al. 2012), may therefore play a role in androgen mediated prostate cancer progression. Interestingly, there is a strong link between mutations in the breast cancer predisposition genes one and two (*BRCA1* and *BRCA2*) and significantly increased prostate cancer risk and increased aggressiveness of the disease (Castro & Eeles 2012). Some mutations result in truncated and dysfunctional

proteins and suggests that the characterisation of alternate forms of protein may be able to differentiate prostate cancer from non-malignant cells.

1.2.3.5 Androgen regulation

Normal development of the prostate gland requires the secretion of growth factors such as insulin-like growth factor, keratinocyte growth factor, fibroblast growth factors and epidermal growth factors from stromal fibroblasts. The synthesis and secretion of these factors is stimulated by androgens (Chatterjee 2003), however, over-stimulation and elevated secretion of growth factors promotes aberrant cell regulation, proliferation or dysfunctional apoptosis of epithelial or stromal cells. Moreover, these growth factors stimulate the development of more aggressive phenotypes of prostate cancer (Lorenzo et al. 2003; Russell, Bennett & Stricker 1998). Cell proliferation and development of primary prostate cancer is initially dependent upon androgen and can be slowed through androgen-ablation therapy, however, the androgen dependence for signalling and proliferation of the cancer cells may diminish. Thus, the cells continue to proliferate as the cancer develops further, despite healthy prostate tissue undergoing atrophy, involution or apoptosis upon androgen deprivation therapy (Montalvo et al. 2000).

1.3 Cancer biomarker discovery

More sensitive and specific markers of prostate cancer are required for the early detection of this disease, to avoid false-positive or false-negative diagnosis and over-treatment. Protein expression, post-translational modifications, mutations and novel isoforms are able to be detected from prostate cancer cells, however high-throughput biomarker discovery has been enabled through analysis of gene and protein expression from gene or tissue microarrays (Butte 2002; Wang, XY et al. 2013).

1.3.1 Proteomic and genomic approaches to biomarker discovery

A number of genes that may be involved in regulatory pathways of cancer biology can be quantitated using microarray technologies, providing avenues for new biomarker discovery. Several studies have previously compared the gene expression profiles of normal, benign and prostate cancer tissues by cDNA microarray analysis. These have revealed a number of differentially expressed genes between the tissue and disease types; however, the use of gene expression markers in diagnosis may be limited by their variable expression between the heterogenic cancers. α-Methylacyl-CoA racemase (*AMACR*), was highly expressed in prostate cancer, and may be used as a diagnostic tool for the disease (Rubin et al. 2002). The expression of *AMACR* is androgen regulated and will vary in a similar manner to hK3 (*KLK3*) through the development of prostate cancer and response to androgen-deprivation therapy (Vaarala et al. 2012). Gene expression profiles may also vary depending on race or geographic location (Yamada et al. 2013), potentially limiting their diagnostic value. In a clinical environment, quantitation of gene expression requires direct sampling of mRNA from the prostate via tissue biopsies, unless sufficiently expressed in free prostate cells, as found with *PCA3*. Therefore, whilst gene expression may provide a prognostic indicator for disease progression, their use as a diagnostic tool is currently limited.

There are many microarray databases for prostate cancer, however there are discrepancies between results from inter-laboratory variations of tissue sample collection and RNA extraction and the continuing improvements in GeneChip® technologies. In particular, laser-capture micro dissection significantly improves the purity of samples obtained from biopsy tissue, reducing the chance of contaminating diseased tissue with normal, healthy tissue (Simone et al. 1998). Further, there are continued advancements in the algorithms

used for GeneChip® image processing, thus older microarray data may benefit from reanalysis that may provide more accurate gene expression profiles. The analysis of multiple microarray datasets is required to validate and establish accurate models of gene expression.

Altered gene expression does not imply an altered level of translated protein product in the cancer, nor permit definitive conclusions on the biological effects from gene up- or down-regulation, however the analysis of gene expression may reveal effects of transcription factor regulation and downstream effects of signalling pathways. Proteomic analysis may yield greater insight into changes of cell phenotype during cancer progression because they are functional effectors that alter the cell's response to stimuli. Alterations in the proteome during disease onset and progression may provide an avenue for the discovery of novel biomarkers and these may be quantifiable via analysis of biofluids or prostate tissue.

Proteome analysis techniques include mass-spectrometry techniques such as electrospray ionisation and matrix-assisted laser desorption/ionisation that may be used in combination with two-dimensional gel electrophoresis. Biofluids are analysed to determine differences in protein abundance and protein modifications between cancer patients and healthy controls. However, the proteins specific to prostate cancer may be masked by the high abundance of non-specific serum proteins, and must be removed by chromatographic methods to reduce interference. More direct proteomic analysis of biopsy tissue and proximal fluid from cell secretions could reduce this masking effect of abundant serum proteins (Li, R et al. 2008). Moreover, prostate-specific proteins and potential marker candidates have previously been identified in urine from prostate cancer patients (Katafigiotis et al. 2012; ; Principe et al. 2012; Theodorescu et al. 2008, ; for a detailed

review of potential candidate markers and sources see Pin, Fredolini & Petricoin 2012). The use of biofluids as a source of cancer markers provides an advantageous method for sustained analysis of protein concentrations that may fluctuate during cancer onset and progression. However, the wide heterogeneity of individual prostate cancers, inter-patient expression variations, and high numbers of non-prostate specific proteins in tissue biofluids complicate and limit expeditious discovery of novel markers. Fortunately, two prostate cancer models, *in vitro* prostate cell cultures and transgenic mouse models, are available that aid discovery, avoiding interminable issues with patient cohorts.

1.3.2 Cell lines as models of prostate cancer

Cell lines are a commonly used model of prostate cancer and have previously been acquired from metastasised prostate cancer tissues in the lymph node, brain and bone or from primary prostate cancer or non-malignant prostate. They have been heavily utilised for prostate cancer biomarker discovery, however there have been significant problems with these studies, in terms of the number and type of prostate cell lines examined. Studies involving multiple non-malignant control cell lines have been limited, despite the number of prostate cell lines available (see for example: Colella, Rita, Goodwyn & Gopal 2002; Gao et al. 2012; Moro, L. et al. 2007; O'Connell et al. 2012; Sardana et al. 2008; Sardana, Marshall & Diamandis 2007). The large number of studies characterising individual prostate cell lines may have actually been detrimental to effective biomarker discovery; as these candidate markers may not be commonly expressed in other prostate cancer cells or have the capacity to discriminate between non-malignant and cancer cells.

There have been endeavours to characterise the growth and invasive capacity of prostate cell lines, together with androgen receptor expression, androgen response and mutation

analysis of the androgen receptor and p53 or TP53 (Sobel & Sadar 2005b, 2005a; van Bokhoven et al. 2003; Webber, Bello & Quader 1996, 1997a, 1997b). Online databases of prostate cell lines are published, but are irregular and not maintained. A recent database[2] details the expression of PSA, androgen receptor, kallikrein 2, and a limited number of other markers, however additions and updates are sporadic. Despite these efforts in cell line characterisation, a concurrent study of multiple non-malignant control and prostate cancer cell lines with reference to gene and protein expression and altered cell biology in prostate cancer has not been performed.

1.4 Lysosomal alterations in cancer

From the mid-20th century, variations in lysosomal biology were recognised to occur during cancer progression. Alterations in lysosomal size and distribution were first observed in chemically-induced carcinomas (Smith & Camilleri 1964), and lysosome membrane composition was shown to be altered upon the genesis of cancer that affected lysosome function (Diengdoh 1967). The induction of skin cancer was shown to increase the activity of the lysosomal proteins cathepsin, β-glucuronidase and acid phosphatase (Shamberger & Rudolph 1967), and lysosomal protein expression was found to be altered during the progression and regression of tumours (Kampschmidt & Wells 1968; Shamberger 1969). These results lead to various hypotheses of the association of altered lysosomal biology in cancer initiation and progression (Allison 1966, 1968, 1974). A review by Allison (1974) detailed several studies with observations of lysosomal protein secretions and increased enzyme activity in cancers that include the lysosomal proteins acid phosphatase, β-glucuronidase, β-N-Acetyl-glucosaminidase, and cathepsin B and

[2] capcelllines.ca/downloads/CaPCellLines_database.xls (accessed December 2012)

cathepsin D (Allison 1974). Moreover, both precursor and mature acid phosphatase are secreted (Waheed & Van Etten 1986), suggesting that altered lysosomal biology affects the trafficking and maturation of lysosomal proteins.

Upon prostate cancer induction in rats, the number and size of lysosomes in epithelial cells of the cancer increased (Aughey, Scott & McLaughlin 1974) and resulted in elevated acid phosphatase serum concentration and extra-lysosomal acid phosphatase staining (Fischer et al. 1979). Acid phosphatase expression increased inversely to androgen concentration (Waheed & Van Etten 1986) and the expression declined during prostate cancer progression and metastasis (Moss, Raymond & Wile 1995), affecting its efficacy as a diagnostic marker.

Lysosomal proteins have been detected in secretions from malignant cells derived from a variety of tissues (Recklies et al. 1980; Sinha et al. 1995a; Sinha et al. 2001; Sinha et al. 1995b; Sloane, Bonnie F. et al. 1982; Troy et al. 2004). The proteinase cathepsin B is of particular importance since its activity was not reduced in less-acidic environments outside of the lysosome, and was therefore active in the extracellular matrix. Increased levels of cathepsin B in prostatic intraepithelial neoplasia and prostate cancer tissue and cell lines have been observed, together with altered localisation to plasma membranes (Colella, Rita, Goodwyn & Gopal 2002; Sinha et al. 2001; Sinha et al. 1995b). Dissection of solid tumour mass has shown that cathepsin B activity was high on the exterior face of malignant breast tissue (Recklies et al. 1980), and was also associated with the plasma membrane in cells from metastatic melanoma (Sloane, B. F. et al. 1986). Cathepsin B has therefore been implicated in extracellular matrix degradation, metastasis and angiogenesis. Cathepsin D has been used as a marker of metastasis in various types of cancer (Rochefort

et al. 2000), with elevated concentrations correlating to metastatic development. Cathepsin D has also been found to promote angiogenesis (Berchem et al. 2002). Despite these associations with cancer, the use of cathepsin D as a diagnostic or prognostic marker is not recommended[3]. Cells transfected with oncogenic *ras* show alterations in both cathepsin B and cathepsin D trafficking to the cell periphery. This trafficking of lysosomes away from the perinuclear region (Demoz et al. 1999; Nishimura, Sameni & Sloane 1998; Sloane, B. F. et al. 1994) has been related to the aggressiveness and invasiveness of malignant cells (Rempel et al. 1994). Cathepsin L expression is mediated by Ras effector pathways, and upregulated in both transformed fibroblasts (Collette et al. 2004) and many different types of cancer (Chauhan, Goldstein & Gottesman 1991). Similarly, the expression of cathepsin H was increased in prostate cancer and cancer of the bladder (Staack et al. 2004). This evidence suggested that lysosomal trafficking might be altered when cells become malignant and that the release of lysosomal proteins into the extracellular environment may be detected and used to detect tumour onset.

Lysosomal acid phosphatase (Henneberry, Engel & Grayhack 1979) and various glycosidases including acid ceramidase have altered expression in a range of cancers, including prostate cancer (Seelan et al. 2000; Severini et al. 1995; Sinowatz et al. 1978). Acid ceramidase was upregulated in prostate cancer tissues and the overexpression of this enzyme has been implicated in a poor response to cancer treatment (Ferlinz et al. 2001). *In vitro*, increased amounts of acid ceramidase have been reported in prostate cancer cells when compared to benign prostatic cells (Seelan et al. 2000). Elevated amounts of membrane-associated acid phosphatase have been reported in PC-3 and LNCaP cell lines,

[3] asco.org/ASCOv2/Practice+%26+Guidelines/Guidelines/Clinical+Practice+Guidelines (accessed January 2013)

but with a reduced level of soluble acid phosphatase secretion (Quintero et al. 2007). The cathepsins (B, D, L, and H) are also reported to be elevated in some prostate cancer cell lines (Chauhan, Goldstein & Gottesman 1991; Friedrich et al. 1999; Waghray et al. 2002), which suggested that prostate cell lines may provide a suitable model for the study of altered lysosome biology.

The expression and secretion of lysosomal hydrolases is one of a number of alterations that may affect or effect the initiation of prostate cancer. The motility of PC-3 cancer cells and communication between the cells was modulated because of disturbed lysosomal function (Colella, R. & Casey 2003). A reduction in direct cell-cell communication may result from impaired trafficking of connexins that pass through the lysosomal pathways to the cell surface (Govindarajan et al. 2002). Mannose-phosphate receptors, which are trafficked through the lysosomal pathway, may reduce PC-3 cell motility upon receptor inhibition (Evans, CP et al. 1997), thereby linking cell signalling and lysosome biology with the altered metastatic potential of cancer cells. Altered lysosomal biology may further induce redox processes and initiate cancer (Tappel 2005) or promote cancer genesis through failures in lysosome-mediated autophagic processes (Jin & White 2007; Levine & Kroemer 2008). While increased amounts of lysosomal hydrolases and changes in lysosomal distribution have been implicated in prostate cancer biology, a detailed study of lysosomal biogenesis has not been performed in prostate cancer.

1.5 Lysosome biogenesis

Lysosome biogenesis is defined as the integration of the structural and functional elements of the endosome-lysosome pathways of a cell, requiring the coordinated synthesis and

transport of soluble and membrane-bound proteins that are directed to specific compartments and result in the formation and maintenance of acidic organelles.

1.5.1 Lysosomes

Lysosomes are acidic, approximately 500 nm diameter, single-membrane-bound cytoplasmic organelles containing soluble hydrolytic proteins, and were named by De Duve (1955) as a derivative from the Greek for "digestive body" (De Duve et al. 1955). The acidity of these organelles enables the degradation of material from the endocytic and autophagic pathways. Lysosomes remove unnecessary or dysfunctional cell components and proteins to maintain a balance of synthesis, degradation and recycling for proper cell differentiation and organ development (Maiuri et al. 2007; Mizushima & Levine 2010).

The limiting membrane of a lysosome is enriched with glycoproteins that are either integral to, or associated with the membrane; thus named lysosome integral membrane proteins (LIMPs) or lysosome-associated membrane proteins (LAMPs). Lysosome-associated membrane protein 1 (LAMP-1) is one of the most abundant membrane proteins on lysosomes (Saftig & Klumperman 2009), enabling these organelles to be distinguished from endosomes by the elevated presence of this protein. Lysosome-associated membrane proteins are critical for the function and trafficking of lysosomes and the expression of LAMPs may determine the susceptibility of cells to the lysosomal cell death pathway and affect their cellular distribution (Saftig & Klumperman 2009). Moreover, lysosomes distribute to the cell periphery away from the microtubule organising centre in LAMP depleted cells (Fehrenbacher et al. 2008). Changes in lysosome distribution (Smith & Camilleri 1964), may therefore be attributed to altered LAMP expression and may provide an avenue for the differentiation of normal and cancer cells if this phenomenon occurs in

prostate cancer. The membrane protein LIMP-2 is involved in the biogenesis and maintenance of endosomes and lysosomes through interactions with fission and fusion machinery and the control of membrane budding or invagination (Eskelinen, Tanaka & Saftig 2003). Overexpression of LIMP-2 results in the formation of large endosome-lysosome hybrid vacuoles and affects trafficking from these compartments (Kuronita et al. 2002). Thus, LIMP-2 and LAMP-1 are ideal candidates for the analysis of altered lysosome biology in prostate cancer.

Lysosomes are a terminal compartment for the biosynthetic and endocytic pathways, and these organelles can be involved in programmed cell death, cell membrane repair and tissue remodelling (Kroemer & Jaattela 2005; Reddy, Caler & Andrews 2001; Vasiljeva & Turk 2008). These functions are mediated through the action of lysosomal proteases, glycosidases, lipases, sulphatases and phosphatases (Schroder, BA et al. 2010). Many soluble lysosomal proteins are involved in proteolysis or hydrolysis and are critical to cell maintenance. The significance of lysosome function is illustrated by a number of diseases that occur upon dysfunction of lysosomal hydrolases (Schroder, BA et al. 2010). A deficiency in β-glucosidase is the cause of Gaucher disease and results in reduced hydrolysis of glucosyl ceramides in lysosomes. Interestingly, the risk of cancer onset is increased in patients with Gaucher disease (Mistry et al. 2013), which may relate to lysosomal dysfunction. The majority of lysosomal hydrolases are synthesised as precursors that are processed into mature active forms in the acidic environment of endosome and lysosome compartments. The hydrolase α-glucosidase requires glycosylation and proteolytic processing for the efficient trafficking and activation of the enzyme, and multiple species of this protein have been observed that have varying molecular weights

and enzymatic activities (Wisselaar et al. 1993) and suggests that the function of these proteases may therefore be affected by altered lysosomal biogenesis.

Other lysosomal proteins that have been investigated in this thesis include the enzyme α-galactosidase A, which hydrolyses alpha-galactosyl moieties from glycolipids and glycoproteins, and can catalyse the hydrolysis of melibiose into galactose and glucose. Loss of α-galactosidase A presents as Fabry disease, and has also been linked to increased incidence or risk of cancer (Pagni et al. 2012). The lysosomal enzyme acid ceramidase is also correlated to increased incidence of cancer when overexpressed. Acid ceramidase hydrolyses the lipid ceramide to free fatty acid and sphingosine, which may be further phosphorylated to form sphingosine-1-phosphate, a signalling lipid implicated in cell proliferation. The localisation and processing of precursor acid ceramidase to an active enzyme in the lysosome requires efficient glycosylation and trafficking by mannose-6-phosphate receptor of the acid ceramidase precursor (Ferlinz et al. 2001). The overexpression of acid ceramidase has been observed in a range of cancers including prostate cancer, and this may result in increased conversion of ceramide to sphingosine-1-phosphate, increasing cell proliferation (Zeidan et al. 2008). In turn, this may affect the expression of cathepsin B and alter its intracellular location (Beckham et al. 2012). The lysosomal proteases cathepsin B and cathepsin D participate in proteolytic cascades and are important components of the apoptotic processes (Brix et al. 2008). Due to their proteolytic activity, cathepsins B and D are often involved in extracellular matrix remodelling during tissue development (Green & Lund 2005; Margaryan et al. 2010). The activity of cathepsins upon secretion from cells may be minimised through inhibition by cysteine peptidase inhibitors (Brix et al. 2008), however the overexpression of cathepsins and their increased secretion may result in aberrant matrix remodelling and result in

increased metastatic potential (Nomura & Katunuma 2005). While increased amounts of lysosomal hydrolases and changes in lysosomal distribution have been implicated in cancer biology, a detailed study of the expression, posttranslational processing and secretion of α-galactosidase A, α- and β-glucosidases acid ceramidase and cathepsins B and D have not been performed in multiple non-malignant and prostate cancer cell lines.

1.5.2 Trafficking of lysosomal hydrolases to the lysosome

Targeting of proteins from the trans-Golgi network to the lysosome occurs directly via the late-endosomal or pre-lysosomal compartment, or indirectly via the plasma membrane and endocytosed into an early-endosome system (Saftig & Klumperman 2009). Newly synthesised lysosomal hydrolases are glycosylated on asparagine residues within the endoplasmic reticulum. The enzyme N-acetylglucosamine-1-phosphotransferase catalyses a two-step process that generates and exposes a recognition signal for mannose-6-phosphate receptors to bind to soluble lysosomal hydrolases. The 46 kDa cation-dependent mannose-6-phosphate receptor mediates trafficking from the trans-Golgi network to the late-endosome/pre-lysosomal compartments (Dahms, Lobel & Kornfeld 1989), whilst the 300 kDa cation-independent mannose-6-phosphate receptor (Griffiths et al. 1988) delivers proteins from the cell surface into endosomes. In addition to trafficking mediated by the two mannose-6-phosphate receptors, two alternative pathways are implicated in delivery of proteins to the lysosome (Coutinho, Prata & Alves 2012b). The integral membrane receptor LIMP-2 binds the ligand β-glucocerebrosidase (β-GC), functioning as a trafficking receptor that acts in a mannose-6-phosphate-independent manner (Reczek et al. 2007). Sortilin is a multifunctional type I transmembrane protein serving as a transport receptor for neurotensin, lipoprotein lipase, and the precursors of sphingolipid activator proteins (Braulke & Bonifacino 2009; Canuel et al. 2008). Dysfunction of receptors and

altered trafficking may result in miss-targeting of lysosomal hydrolases and secretion of these into the extracellular environment. This may result in the accumulation of immature 'proenzymes' such as pro-cathepsin B and pro-cathepsin D that have not undergone appropriate maturation in acidic organelles (Linke, Herzog & Brix 2002). Moreover, the extent of dysfunctional trafficking may be demonstrated by the amount of mature or immature lysosomal hydrolases that are secreted from a cell. Secretion of lysosomal proteins may be involved in cancer pathogenesis, particularly through their proteolytic activity and the degradation of the extracellular matrix that facilitates metastasis (Nomura & Katunuma 2005). The presence of cathepsins in the extracellular matrix may also induce activation of growth factors and activate MAPK-ERK signalling pathways and enhance proliferation (Kallunki, Olsen & Jaattela 2012). Understanding the cell biology of prostate cancer and investigation of lysosomal protein secretion from prostate cell lines is important in order to develop new approaches for the early diagnosis, prognosis and treatment for prostate cancer patients.

The targeting of receptor-ligand complexes from the biosynthetic compartment is mediated by vesicular trafficking from the trans-Golgi network to the lysosomes. The receptor-ligand complex is packaged into clathrin-coated vesicles (Braulke & Bonifacino 2009) that then release the clathrin and translocate to endosome compartments using a microtubule network and kinesin or dynein/dynactin motor proteins (Jordens et al. 2005). The transport vesicles fuse with the endosome and the acidity of this compartment induces ligand dissociation from the receptor (Coutinho, Prata & Alves 2012a). Maturation from the late endosome to the lysosome involves a fusion or 'kissing' event (Bright, NA, Gratian & Luzio 2005) mediated by tethering of SNARE complexes between late endosome and lysosome, forming a hybrid organelle (Luzio, J. Paul et al. 2003; Luzio, J. P. et al. 2000). The

interactions and biogenesis of the endosome and lysosome compartments may be regulated by both LIMP-2 and interactions with the fusion machinery. The altered expression or trafficking of LIMP-2 impaired vesicular budding from endosomes and altered the morphology and composition of hybrid endosome-lysosome organelles (Kuronita et al. 2002; Kuronita et al. 2005). Following fusion events, the hybrid organelle is recovered to a late endosome and lysosome through the condensation of material via a combination of decreasing pH via V-ATPase proton pumps, increasing concentration of Ca^{2+} ions and the action of clathrin and adaptor proteins (Bright, N et al. 1997; Huotari & Helenius 2011; Luzio, J. P., Bright & Pryor 2007; Luzio, J. P. et al. 2000). The maintenance of compartment acidity is required for the efficient dissociation of receptor/ligand complexes and posttranslational modification and functional activity of enzymes (Pillay, Elliott & Dennison 2002). However, lysosomal cathepsins secreted into the extracellular environment maintain some of their hydrolytic activities despite its more pH-neutral environment, and induce extracellular matrix remodelling, which may be critical to the genesis and progression of cancer (Roshy, Sloane & Moin 2003). Aberrantly secreted lysosomal hydrolases may therefore be ideal candidate biomarkers for cancer.

1.5.3 The endosome network and endocytic function

Early endosomes are mildly acidic (pH 6) dynamic compartments of tubules and vacuolar domains with a mosaic of membrane subdomains that are enriched in specific proteins. These membrane subdomains provide a mechanism for protein sorting, and generate vesicles that are transported to the plasma membrane, recycling endosomes or trans-Golgi network (Huotari & Helenius 2011).

Trafficking via the endocytic pathway requires the transport of material from the plasma membrane to lysosomes through intermediate endosomal compartments that mature from early to late endosomes and lysosomes by sequential fusion and vesicular budding events (reviewed by Saftig & Klumperman 2009). An endocytic event results in the internalisation of membrane and the formation of a small transport vesicle, or pre-early endosome (Figure 1.5). The GTPase Rab5A localises with the cell surface on clathrin-coated pits and recruits Appl1. Appl1 may bind directly to Rab5A, the lipid bilayer or via membrane receptors such as the Epidermal Growth Factor Receptor (Zhu et al. 2007). Subsequently during the maturation of these vesicles, Appl1 is displaced by phosphatidylinositol 3-phosphate (PI3P) (Li, J et al. 2007; Zoncu et al. 2009). The combination of Rab5A and PI3P provides moieties for the efficient binding of effectors such as Early Endosomal Antigen 1 (EEA1) and Rabenosyn-5 (Zerial & McBride 2001) and this occurs during maturation to an early endosome, which is characterised by the presence of Rab5 and Appl1 or EEA1 proteins. Appl1 endosomes are important for the assembly of signalling-complexes that regulate the MAPK and Akt signalling pathways. Altered Appl1 recruitment or displacement can therefore result in aberrant signalling (Lin et al. 2006; Schenck et al. 2008). The phosphorylation of PI3P and expression of Rab5 are linked in such a way that depletion of PI3P may result in the loss of EEA1, re-recruitment of Appl1 and in the expansion and enhanced signalling from early endosomes (Zoncu et al. 2009). Maturation from an early to a late endosome involves a Rab5/Rab7 switch, whereby Rab5 hydrolysis of GTP enables the extraction from the endosome membrane and subsequent recruitment of Rab7 and associated subunits (Rink et al. 2005). The recruitment of Rab7 is critical for trafficking from the late endosome to the lysosome, and the loss of Rab7 results in accumulation of

endocytosed receptors and an enlarged late endocytic compartment (Vanlandingham & Ceresa 2009).

The trafficking of receptors from the early endosome to the cell surface is enabled via recycling endosomes. Early endosomes mature into sorting endosomes by the inclusion of Rab4 on the Rab5-localised early endosome membrane (Grant & Donaldson 2009). This enables rapid recycling to the cell surface, a process that is regulated by Rab4 (van der Sluijs et al. 1992). A slower recycling pathway is attained through the recycling endosome compartment, characterised by Rab4 and Rab11 GTPases (Eggers et al. 2009). The Rab11 GTPase regulates trafficking through this compartment (Ullrich et al. 1996), and is involved in vesicular delivery of cargo to the cell surface. Receptor recycling through these endosome compartments enables the regulation of intracellular signalling pathways through the control of the number of receptors present on the cell surface.

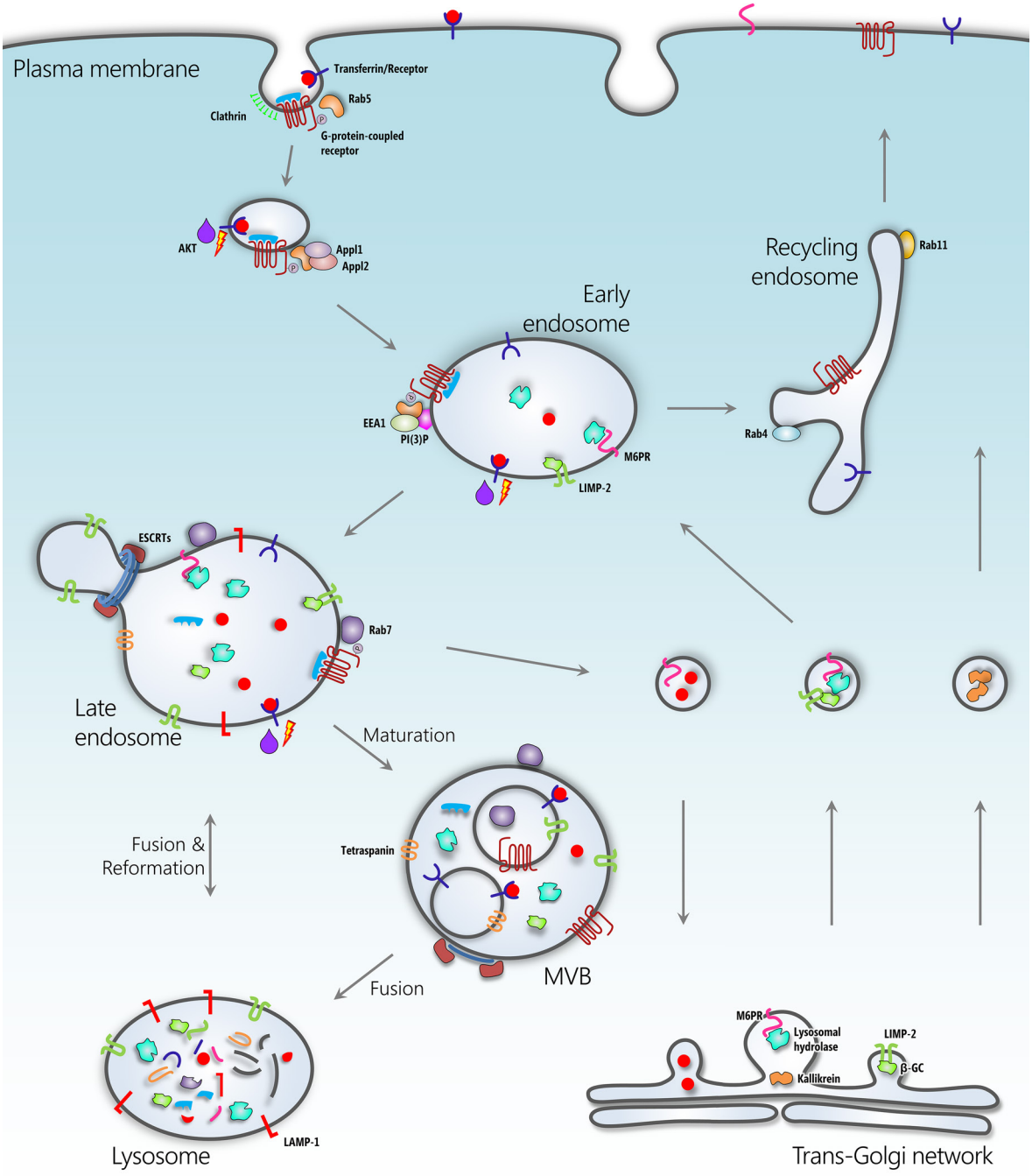


Figure 1.5 – Lysosome biogenesis, lysosomal hydrolase and receptor trafficking. Ligand-bound receptor is endocytosed by clathrin-independent or dependent mechanisms. Receptor-mediated signalling (⚡) may occur from early endosome or late endosome compartments.

The “endosomal sorting complex required for transport” (ESCRT) complexes are associated with the late endosomal membrane and are capable of catalysing the invagination of limiting membrane to create intraluminal vesicles (exosomes), with the resulting organelle classified as a multivesicular body (Babst 2011; Hurley 2008). Exosomes are small membrane vesicles that range in size from approximately 40-100 nm that can be either released or secreted from different cell types (Morelli et al. 2004; Théry, Zitvogel & Amigorena 2002). Exosome-like vesicles derived from epithelial cells of the prostate have been called prostasomes. The exosome release process (Figure 1.6) initially involves specific sorting of internal vesicles to the lumen of endosomes to form a multivesicular endosome. The fusion of the endosome limiting membrane with the plasma membrane results in the vesicular release of exosomes and endosome contents into the extracellular milieu.

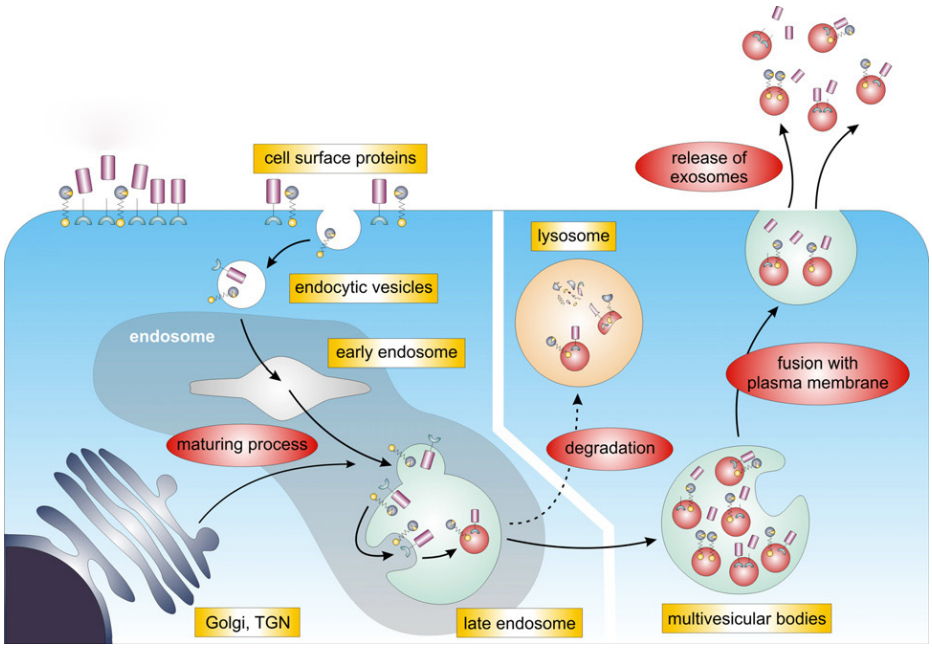


Figure 1.6 – Exosome vesicle formation and release (adapted from Keller et al. 2006).

Proteins most commonly associated with exosomes are those from the tetraspanin family, namely CD9, CD63, CD81 and CD82 (Bard et al. 2004; Escola et al. 1998; Olver & Vidal 2007; Van Niel et al. 2003). Other common exosomal membrane proteins include the major histocompatibility complexes I and II, which are involved in antigen presentation and T-cell stimulation (Skokos et al. 2001; Théry, Zitvogel & Amigorena 2002). Exosomes also contain cytosolic proteins such as Annexin II, Rab5 and Rab7, tumour susceptibility protein TSG101 and heat shock proteins HSP70 and HSP90 (Gruenberg & Maxfield 1995; Lancaster & Febbraio 2005; Olver & Vidal 2007). The increased release of exosomes from cancer cells (Cocucci, Racchetti & Meldolesi 2009) may induce aberrant signalling (Yang, C & Robbins 2011) and suppression of immune responses (Liu, C et al. 2006), and be involved in cancer progression.

Prostate cells release exosome vesicles into both semen and urine and these biological fluids may contain cancer-associated markers such as kallikrein 3 and prostate cancer antigen 3 (Nilsson et al. 2009). The characterisation of secreted exosomes from prostate cancer cells may provide a valuable method for the detection of cancer and evaluation of treatment responses (Duijvesz et al. 2011; Mitchell et al. 2009). Despite the recognised increase in exosome production and release from cancer cells, these processes have not been fully characterised in prostate cancer. LNCaP and DU-145 cell lines have been utilised as a source of exosomes for comparison of urinary-derived exosomes from prostate cancer patients and healthy donors (Mitchell et al. 2009). Moreover, CDCP1 and CD151 proteins were characterised as potential cancer biomarkers from exosome vesicles derived from the PC-3 prostate cancer cell line (Sandvig & Llorente 2012). However, the determination of potential biomarkers was obtained from a single control cell line and cancer cell line and not validated from additional sources. Similarly, characterisation of the anti-angiogenic

potential of prostasomes has been performed using three cancer cell lines and a single non-malignant control cell line (Babiker et al. 2010). The limited use of non-malignant prostate cell lines for biomarker studies is widespread, despite the high number of cancer cell lines utilised (Hosseini-Beheshti et al. 2012). The characterisation of prostate derived exosomes may aid in biomarker discovery and provide a potential avenue for cancer therapy.

1.5.4 Receptor-mediated signalling pathways

Androgen receptor signalling is critical for the development of normal prostate and is involved in the progression of prostate cancer, particularly metastatic cancers. The androgen receptor is a cytoplasmic protein, tethered to cytoskeletal proteins via heat shock proteins that modulate androgen receptor conformation. Androgen hormones entering the cell, bind the androgen receptor and induce a conformational change; resulting in receptor dissociation from the heat shock proteins, enabling interaction with co-regulators that bind to the newly exposed nuclear localisation signal. Consequently, the androgen receptor is targeted to the nucleus and binds specific androgen response elements, recruiting histone acetyltransferases and transcription machinery and transcribing specific genes (Bennett et al. 2010). Androgen receptor activity is mediated by the activity of mitogen activated protein kinases (MAPKs), extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases (JNKs), Akt and p38 (Rochette-Egly 2003). Aberrant activation of these kinases, which could occur through endosome-mediated signalling, may therefore activate the receptor in an androgen-independent manner, inducing androgen-independent signalling leading to cell proliferation.

1.5.5 Signalling from the endocytic pathway

The activation of MAPK, ERK, JNK or Akt signalling molecules occurs via receptor-mediated pathways from the cell-surface, where upon ligand-induced activation, receptors undergo rapid clathrin-dependent endocytosis (Murphy et al. 2009). This is mediated by ubiquitination that serves as a targeting signal for clathrin and exposes internalisation motifs to adapter protein 2 (Schmid 1997). Adapter proteins function as regulators for internalising some receptors such as the transferrin receptor (TfR) (Lamaze et al. 1993). Internalised receptors are sorted from Rab5-positive endosomes to the recycling or degradation pathways. The internalised receptors are recycled to the plasma membrane by a number of mechanisms that are Rab4-dependent or route through Rab11-positive recycling compartments (Sorkin & von Zastrow 2009). The internalised receptors may also remain in endosomes that mature into late endosomes and multivesicular bodies. The assimilation of membrane receptors into intraluminal vesicles attenuates receptor signalling, and enables their degradation upon fusion of the multivesicular body with lysosomes (Futter et al. 1996). The various trafficking routes through the recycling or late endosomes therefore enable control of cell-surface receptor content and regulation of signalling. Endocytosis of the epidermal growth factor receptor is altered in androgen receptor-positive prostate cancer cell lines (Bonaccorsi et al. 2007), however the endosomal network downstream has not been analysed in multiple non-malignant and prostate cancer cells. Moreover, receptor and signal-related kinases have been observed in a multitude of endosomal pools (Sadowski, Pilecka & Miaczynska 2009), and the signalling activity may be maintained until the ligand-receptor complex dissociates in a late endosome or lysosome, or are incorporated into multivesicular bodies (Sorkin & Von Zastrow 2002; Wang, Y et al. 2002). This suggests that endosome and lysosome function

and biogenesis may be critical to the regulation of cell signalling and homeostasis that may be disturbed in prostate cancer cells.

1.6 Rationale, hypothesis and aims

1.6.1 Rationale

Current methods for prostate cancer detection are limited in terms of both specificity or sensitivity to the disease and the ability to adequately diagnose patients at risk of progressive disease and mortality. Thus, a method to divide patients into prognostic subgroups and to identify patients who have comparable biochemistry and survival is yet to be developed. While increased amounts of lysosomal hydrolases and changes in lysosomal distribution have been implicated in prostate cancer biology, a detailed study of lysosomal biogenesis has not been performed in prostate cancer. The characterisation of lysosomal biology in prostate cancer may open a new avenue of investigation for the development of more specific markers to the disease. These may improve the diagnostic capacity of current detection tests through increased specificity to the disease compared to prostatitis or benign prostatic hyperplasia, to aid in the prognosis and treatment strategies of prostate cancer patients.

1.6.2 Hypothesis

Lysosome biogenesis is altered in prostate cancer. Endosome and lysosome proteins may be used to discriminate between normal and prostate cancer cells providing a diagnostic or prognostic marker for prostate cancer patients.

1.6.3 Aims

1. Investigate endosome and lysosome proteins in prostate cancer cell lines.

2. Identify potential biomarkers that have the capacity to distinguish prostate cancer from non-malignant control cell lines.

3. Confirm that any alterations in marker expression in prostate cancer cell lines are also observed in microarray patient cohorts.

4. Investigate any biomarker association with patient clinical outcomes

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Antibodies and fluorescent probes

Specific concentrations of primary antibodies and secondary antibody conjugates used for Western blotting analysis and immune fluorescence are detailed in Table 2.1 and Table 2.2. Table 2.3 details the properties of fluorescent-conjugate antibodies used in confocal microscopy analysis. Where dual labelling was performed using two primary antibodies of the same host-species, Zenon® IgG labelling kits (Life Technologies Australia Pty Ltd., Victoria, Australia) were used to label the primary antibodies directly, with the labelling protocol carried out as per manufacturers' instructions.

Table 2.1 – Primary and secondary antibodies used in Western blot analysis

Antibody	Working concentration	Source cat#
M- Cathepsin B	0.25 µg/mL	ab58802
M- Cathepsin D	5 µg/mL	ab6313
Rb- Acid ceramidase	1 µg/mL	ab74469
Sh- α-Glucosidase	1 µg/mL	In-house
Sh- β-Glucosidase	1 µg/mL	In-house
Sh- α-Galactosidase A	1 µg/mL	In-house
M- Prostatic acid phosphatase	1 µg/mL	ab75704
Rb- hK2	1 µg/mL	ab40948
Rb- hK3	1 µg/mL	ab40949
Rb- hK4	1 µg/mL	ab40950
Rb- hK15	1 µg/mL	ab40961
M- TFEB	1 µg/mL	ab56330
Rb- TfR1	1 µg/mL	ab108985
Rb- TfR2	1 µg/mL	ab80194
Sh- LIMP-2	5 µg/mL	In-house
Rb- Appl1	0.4 µg/mL	ab95195
Rb- Appl2	0.4 µg/mL	ab95196
G- Rab5A	1 µg/mL	R9704
G- EEA1	1 µg/mL	sc-6415
Rb- Rab4	1 µg/mL	ab13252
G- Rab7	1 µg/mL	Sc-6563
M- LAMP-1 BB6	1 µg/mL	In-house
Rb- AKT	2 µg/mL	In-house
Rb- AKT-P	2 µg/mL	In-house
M- GAPDH HRP	1/20000	G9295
G- α-Rabbit HRP	1/2000	A6154
G- α-Mouse HRP	1/2000	A4416
D- α-Sheep/Goat HRP	1/2000	AB324P

Table 2.2 – Primary antibodies and fluorophores used in immune fluorescence assays

Antibody	Working concentration	Source cat#
Rb- TfR1	5 µg/mL	ab108985
Rb- TfR2	5 µg/mL	ab80194
Sh- LIMP-2	10 µg/mL	In-house
Rb- Appl1	2 µg/mL	ab95195
G- Rab5A	4 µg/mL	R9704
G- EEA1	4 µg/mL	sc-6415
Rb- Rab4	4 µg/mL	ab13252
G- Rab7A	4 µg/mL	Sc-6563
M- LAMP-1 BB6	5 µg/mL	In-house
Rb- TGN46	10 µg/mL	ab50595
LysoTracker® 488	5 µM (1/200)	L7535
Transferrin 633	5 µg/mL	T23362
Phalloidin 488	1/100	A12379
D- α-Mouse Alexa fluor® 488	1/250	A11055
D- α-Sheep Alexa fluor® 488	1/250	A11015
D- α-Goat Cy3	1/250	AP180C

D: Donkey; G: Goat; M: Mouse; Rb: Rabbit; Sh: Sheep

Table 2.3 – Fluorescent probes used in laser-scanning confocal microscopy

Fluorophore	Excitation maxima (λ)	Emission maxima (λ)	Detection spectrum (nm)
DAPI	358	461	407 – 471
Phalloidin Alexa Fluor® 488	495	518	500 – 560
Alexa Fluor® 488	495	519	500 – 550
Zenon® 488	495	519	500 – 550
LysoTracker® Green DND-26	504	511	505 – 560
LysoTracker® Red DND-99	577	590	590 – 700
Zenon® 568	578	603	570 – 630
Cy3	550	570	570 – 630
Alexa Fluor® 633	632	647	650 – 730
Transferrin Alexa Fluor® 633	632	647	650 – 730

2.2 Cell Culture and extract preparation

2.2.1 Cell lines

The non-malignant prostate cell lines PNT1a and PNT2, and prostate cancer cell lines 22RV1 and LNCaP clone FCG were obtained from the European Collection of Cell Cultures via CellBank Australia (Children's Medical Research Institute, New South Wales, Australia). The non-malignant prostate cell line RWPE-1, and prostate cancer cell lines CaHPV10 and DU-145, were obtained from the American Tissue Culture Collection via Cryosite (Cryosite Ltd., New South Wales, Australia). These cell lines are absent from the List of Cross-Contaminated or Misidentified Cell Lines, version 6.8 (9th March 2012; Capes-Davis et al. 2010). The cell lines RWPE-1, PNT1a and PNT2 were used in this study

to represent normal non-malignant epithelial-cell phenotypes, and have been adapted and immortalised with viruses. Specifically, human papilloma virus-18 (HPV-18) was used to immortalise the RWPE-1 cell line (Bello et al. 1997), and Simian vacuolating virus 40 (SV40) to immortalise PNT1a and PNT2 cell lines (Berthon et al. 1995). The cancer cell line CaHPV10 was derived from a primary adenocarcinoma of the prostate immortalised with HPV-18. This cell line is not androgen responsive. The 22RV1 cancer cell line was derived from a xenograft that had been serially propagated in mice after castration-induced regression and relapse of a parental, androgen-dependent xenograft (Sramkoski et al. 1999). The cell line expresses androgen receptor however its proliferation is unresponsive to androgen stimulation (van Bokhoven et al. 2003). The cancer cell line LNCaP was previously derived from a lymph node metastasis of prostate adenocarcinoma (Horoszewicz et al. 1983). The LNCaP cell line is androgen responsive. The cancer cell line DU-145 was previously derived from a moderately-differentiated brain metastasis of prostate adenocarcinoma (Stone et al. 1978). They are epithelial cells, and unresponsive to androgen stimulation.

2.2.2 Culture conditions

PNT1a, PNT2 and 22RV1 cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco®, Life Technologies), supplemented with 10 % (v/v) foetal calf serum (FCS) (In Vitro Technologies Pty Ltd., Victoria, Australia) and 2 mM L-glutamine (Sigma Aldrich Pty Ltd., New South Wales, Australia). RWPE-1 and CaHPV10 cell lines were maintained in Keratinocyte Serum-Free Media containing L-glutamine (Gibco®), supplemented with the supplied human recombinant Epidermal Growth Factor 1-53 and Bovine Pituitary Extract. The DU-145 cell line was maintained in Minimum Essential Medium (Gibco®) and supplemented with 2 mM L-glutamine and 10 % (v/v) FCS. The

cancer cell line LNCaP was maintained in RPMI-1640 media supplemented with 2 mM L-glutamine, 10 % (v/v) FCS, 10 mM HEPES (Life Technologies) and 1 mM sodium pyruvate (Sigma Aldrich). Cells were incubated in a humidified incubator at 37 °C with 5 % CO_2 in a Sanyo MCO-17AI CO_2 Incubator (Sanyo Electric Biomedical Co., Ltd., Osaka, Japan).

Cells were passaged at approximately 90 % confluence, washed with sterile DPBS (Sigma Aldrich) and trypsinised using Trypsin-EDTA solution containing 0.12 % (w/v) trypsin, 0.02 % (w/v) EDTA (SAFC®, Sigma Aldrich) until dissociated from the culture flask surface. Trypsin was neutralised with growth media containing 10 % FCS. RWPE-1 and CaHPV10 cell lines cultured in serum-free media were pelleted at 250 *g* for five minutes and washed with DPBS to remove foetal calf serum. Cells were passaged at a 1:10 to 1:20 ratio for general maintenance.

For long-term storage, cell lines were stored in liquid nitrogen. Briefly, cell cultures were passaged as normal and trypsin was inactivated with 10 % (v/v) FCS. Cells were pelleted by centrifugation at 250 *g* for five minutes and resuspended in their respective culture media containing 10 % (v/v) FCS and 10 % (v/v) DMSO (Sigma Aldrich). One millilitre aliquots of cell suspension were stored at -80 °C overnight in an isopropanol freezing container (Nalgene, Thermo Fisher Scientific Australia Pty Ltd., Victoria, Australia) prior to long-term storage in liquid nitrogen.

2.2.3 Sample preparation

2.2.3.1 Cell extract and conditioned media preparation for immune blotting

Cell lines were seeded in T75 flasks and incubated at 37 °C and 5 % CO_2. At a confluence of 80–90 %, culture media was aspirated, cells washed once with DPBS, and cells incubated for 48 hours with serum-free media.

Total cell protein was extracted using 20 mM Tris (pH 7.0), 500 mM sodium chloride and 2 % (w/v) SDS, diluted in Milli-Q H_2O (TNS buffer). Following 48 hours of cell line incubation, cell culture media was aspirated and cells washed twice with room-temperature DPBS. Eight hundred microlitres of TNS buffer was added to the culture flask and rotated to cover cell-layer in its entirety. Cells were harvested by using a rubber cell scraper, and the resulting extract transferred to a 1.5 mL tube. Extract was immediately heated to 65 °C for five minutes and sonicated at high frequency for one minute. To obtain a more homogenous lysate, cell material was passed through a 25-guage (0.65 mm) syringe needle, six times. Additional TNS buffer was added, where required, to produce a less viscous lysate. Samples were stored at -20 °C. Total protein was quantified using a bicinchoninic acid assay (Micro BCA kit, Pierce, Thermo Scientific Pty Ltd., Rockford, IL, USA) according to the manufacturer's instructions. Samples were quantified using a Wallac Victor™ optical plate-reader and Workout software v2.0 (Perkin-Elmer Pty Ltd., Victoria, Australia) using a 5-point parameter standard-fit curve.

Conditioned media collected at time of cell harvesting and previously stored at -80 °C was thawed, and 33 mL aliquots centrifuged at 1000 *g* for 10 minutes at 4 °C to remove any cell debris. Thirty millilitres of supernatant was transferred to a 50 mL conical tube and sodium deoxycholate (Sigma Aldrich) added to a final 0.02 % (v/v) concentration. Following a

brief vortex, the samples were kept on ice for 30 minutes. A 100 % (w/v) solution of trichloroacetic acid (Sigma Aldrich) was added to a final concentration of 15 % (v/v), agitated by vortex for 30 seconds, and incubated on ice for two hours to precipitate protein from the culture media. Precipitated protein was collected by centrifugation of samples at 5,500 *g* for 30 minutes, with supernatant containing trichloroacetic acid and contaminant waste aspirated. The protein pellet was washed with 4 mL ice-cold (-20 °C) acetone by vortex and incubated at room temperature for five minutes. The sample underwent a further centrifugation at 5,500 *g* for 30 minutes and the acetone wash repeated. After a final centrifuge step, the supernatant was aspirated, and conical tube containing protein pellet was dried under a slow stream of nitrogen gas. Each protein pellet, produced from 30 mL media, was resuspended in a 135 µL 1× SDS-sample buffer/PBS solution, and stored at -20 °C for use later in Western blot assays.

2.2.3.2 Cell extract preparation for quantitative analysis of gene expression

Cell lines were cultured in triplicate in 75 cm^2 flasks as described for immunoblotting, with serum deprivation for 48 hours. Media was aspirated and flasks washed with DPBS. One millilitre TRI reagent® (Applied Biosystems Pty Ltd., Victoria, Australia) was added to each T75 flask and scraped using a rubber cell-scraper and transferred to a 1.5 mL tube. Samples were stored at -80 °C until further required.

2.2.3.3 Cell culture preparation for immune fluorescence analysis by confocal microscopy

Cell cultures were passaged as described in section 2.2.2, seeded at low density on 22 mm Number 1 glass coverslips (Menzel-Gläser, Thermo Fisher Scientific Australia Pty Ltd., Victoria, Australia) and incubated in culture media for 48 hours at 37 °C, 5 % CO_2. After 48 hours, culture media was aspirated and cells fixed using 4 % (v/v) formaldehyde/PBS

solution for 20 minutes at room temperature. Fixed cells were further permeabilised with 0.1 % (v/v) Triton-X in PBS for 10 minutes.

2.3 Quantitative analysis of protein expression

2.3.1 SDS-PAGE and transfer

Ten micrograms of total protein from cell lysates was heat-denatured for five minutes at 100 °C in sample buffer (NuPAGE® LDS Sample Buffer (4×), Life Technologies) and reducing agent (50 mM dithiothreitol; NuPAGE® Sample Reducing Agent (10×)). Samples were separated using a 10 % 15-well, or 12 % 17-well SDS-PAGE pre-cast gel in an XCell SureLock™ Mini-Cell electrophoresis system, buffered with SDS-PAGE running-buffer (Life Technologies) and electrophoresed at 120 V (fixed, with variable current), for 1.5 hours. Following electrophoresis, protein was transferred to polyvinylidene difluoride (PVDF) membranes (Polyscreen®, Perkin-Elmer) at 35 V for one hour.

2.3.2 Immunoblotting and detection

Membranes were blocked for 1 hour at room temperature using 5 % (w/v) skim-milk solution in TBS-tween (0.1 % (v/v)) and incubated with the appropriate antibody (Table 2.1) diluted in 5 % (w/v) skim-milk overnight at 4 °C with gentle agitation. Following incubation with the primary antibody, membranes were washed thrice with TBS-tween for five minutes at room temperature. A horseradish-peroxidase conjugate secondary antibody (Table 2.1), diluted to 1/2000 in 5 % (w/v) skim-milk solution, was incubated with the PVDF membrane for one hour at room temperature with gentle agitation.

Membranes were washed three times with TBS-tween for five minutes, followed by antibody detection using electro chemiluminescence. Membranes were incubated for one minute in the dark using Novex® electro chemiluminescent substrate reagent kit (Life

Technologies), and visualised using an ImageQuant™ LAS 4000 imager, software version 1.2.0.101 (GE Healthcare Bio-Sciences Pty Ltd., New South Wales, Australia). A GAPDH loading control was detected using a goat anti-GAPDH HRP conjugate (Sigma Aldrich) at a 1:20,000 dilution in 5 % (w/v) skim-milk. GAPDH was detected on the same blot as the target protein except where molecular weights of GAPDH and the protein would potentially conflict, in which case PVDF membranes were stripped of primary antibody and re-probed with anti-GAPDH HRP conjugate (detailed in 2.3.3 below).

Western blots were performed in triplicate from three independent gels (n = 3) and images quantified using AlphaViewSA™ software v3.0.0.0 (ProteinSimple, Santa Clara, California). Lane-analysis was performed using the software to determine molecular weights of detected protein. The amount of protein was quantified based on signal density and size of band and normalised to GAPDH. A clustered linear-regression or Kruskal–Wallis rank sum statistical method was used to determine significance of detected protein amounts between control and cancer cell lines. Tests were performed using Stata/SE v11.2 (StataCorp LP, Texas, U.S.A). Significant results showed greater than 95 % confidence ($P \leq 0.05$).

2.3.3 Membrane stripping

Following primary-antibody detection, PVDF membranes were stripped and probed for GAPDH. PVDF membranes were washed twice in a stripping buffer containing 0.02 M Tris, 0.5 M NaCl, 2 % (w/v) SDS and 100 mM β-mercaptoethanol in Milli-Q H_2O. Membranes were agitated vigorously with the stripping buffer at 55 °C for 30 minutes and further washed with vigorous agitation three times with TBS-tween for 15, 20 and 30 minutes at room temperature. The PVDF membranes were blocked with 5 % (w/v) skim-

milk in TBS-tween for 1 hour at room temperature and the detection of GAPDH performed using 1:20,000 dilution of HRP-conjugated anti-GAPDH antibody.

2.4 Quantitative analysis of gene expression

2.4.1 RNA extraction

Cells frozen with TRI reagent® (Applied Biosystems) were thawed and incubated at room temperature for five minutes. Two hundred microlitres of chloroform was added to each millilitre of TRI reagent® used in cell harvesting. Samples were shaken vigorously for one minute, and stood at room temperature for three minutes and then centrifuged for 15 minutes at 16,000 *g*, at 4 °C.

RNA extraction was performed using an RNeasy® mini kit following the manufacturer's instructions (Qiagen Pty Ltd., Victoria, Australia). The upper aqueous phase was transferred to a fresh tube and mixed with an equal volume of 70 % (v/v) ethanol, diluted in RNase-free H_2O. Seven hundred microlitres of sample was transferred to an RNeasy® purification column (Qiagen) and centrifuged for 20 seconds at 16,000 *g*. After centrifugation, the flow-through was discarded and the remaining sample/ethanol mix loaded onto the Qiagen column and centrifuged at 16,000 *g* for 20 seconds. Three hundred and fifty microlitres of buffer RW1 (proprietary, Qiagen) was pipetted onto the spin-column membrane and column centrifuged for 15 seconds at 16,000 *g*.

Samples were treated with DNAse I to remove genomic DNA following manufacturer's instructions (Life Sciences Pty Ltd., Victoria, Australia). DNAse I stock solution was diluted 1:7 with buffer RDD (proprietary, Qiagen) of which 80 µL DNAse I solution was then directly applied to the spin-column membrane and incubated for 15 minutes at room temperature.

A further 350 µL buffer RW1 was added onto the column and centrifuged for 15 seconds at 16,000 *g* followed by two washes using 500 µL buffer RPE (proprietary, Qiagen). To remove any remaining buffer, the column was centrifuged for 2 minutes at 16,000 *g* followed by a further one minute at 16,000 *g*. Elution of each sample was achieved by application of 50 µL of RNase-free H_2O directly to the spin-column membrane, followed by centrifugation for one minute at 16,000 *g*. Eluted RNA samples were stored at -80 °C.

The concentration of extracted RNA was determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Australia Pty Ltd., Victoria, Australia) at 260 nm. The 260/280 nm and 260/230 nm ratios were assessed to ensure samples were free from protein and DNA contamination, where a ratio greater than 1.6 indicates a sample free of contamination.

2.4.2 cDNA synthesis

Complementary DNA (cDNA) for use in qRT-PCR was prepared using the High Capacity RNA-to-cDNA Kit following manufacturer's instructions (Life Technologies). For a 20 µL reaction containing 2 µg of RNA, 10 µL master-mix buffer and 1 µL of reverse transcriptase enzyme was added, with the volume made to 20 µL with RNase-free H_2O. Amplification was performed using a DNA Engine® Peltier Thermal Cycler (Bio-Rad Laboratories Pty, Ltd., New South Wales, Australia), with incubation of the reaction mix for one hour at 37 °C followed by 95 °C for five minutes and then chilled to 4 °C. cDNA samples were stored at -20 °C until required.

2.4.3 Primer design

Primer sequences, detailed in Table 2.4 (page 53), were obtained from either published literature, Harvard PrimerBank[4], or designed using NCBI Primer BLAST[5]. Primers selected from the Harvard PrimerBank were chosen based on criteria of the final amplicon-size being less than 150 base-pairs, that their melting temperature be near 60 °C and where possible they extended across an exon-exon junction. Primers designed using Primer-BLAST also conformed to these criteria. Oligonucleotides were purchased from GeneWorks Pty Ltd., Adelaide, South Australia.

2.4.4 Quantitative RT-PCR

Ten microlitre reaction mixtures were set up containing 5 µL Power SYBR® Green PCR Master Mix (Life Technologies), 0.5 µL each of 10 nM forward and reverse primer, 2 µL cDNA sample diluted to 1:25 with DEPC-treated H_2O, and made up to 10 µL with 2 µL DEPC-treated H_2O. Reactions were plated into 96-well plates (Life Technologies) in triplicate, with each plate containing serial dilutions of a reference cDNA sample (from the LNCaP cell line) for the target-gene and endogenous-gene standard curves, to control for reaction efficiency. qPCR was performed using a 7500 Fast Real-Time PCR System (Life Technologies) using ABI 7500 software v2.0.2. Cycling conditions for all targets comprised; 50 °C for 2 minutes, 95 °C for 10 minutes to activate enzyme and denature cDNA, followed by 40 cycles of a 95 °C 15-second denaturation step and a 60 °C 60-second extension and signal-acquisition step. Melt-curves were produced following each run to confirm the absence of primer-dimers or product and other contaminants.

[4] pga.mgh.harvard.edu (accessed 2011 – 2012)
[5] ncbi.nlm.nih.gov/tools/primer-blast (accessed 2011 – 2012)

Cycle threshold values were derived at a threshold level of 0.35, in the exponential phase of amplification and above baseline noise. The relative amount of gene expression from each sample was derived by calculation of cycle threshold values versus standard curves produced from serially-diluted standard material. Reaction efficiencies, calculated from the slope of a linear trend-line plotted from diluted standards, were between 90 and 110 %. Each target was assessed in triplicate on a single plate, with triplicate biological replicates run independently. Mean gene expression was derived from the mRNA amount on each replicate plate; with each single plate providing a mean cycle threshold and mRNA amount from replicate wells.

Table 2.4 – Primer sequences used in qPCR

Gene		Sequence (5' – 3')	GeneBank ID (NM_)	Primer position	Source
GAPDH	F	TGCACCACCAACTGCTTAGC	002046.3	556 – 575	(Sardiello et al. 2009)
	R	GGCATGGACTGTGGTCATGAG		642 – 622	
LAMP1	F	ACGTTACAGCGTCCAGCTCAT	005561.3	608 – 628	(Sardiello et al. 2009)
	R	TCTTTGGAGCTCGCATTGG		685 – 667	
SCARB2 (LIMP2)	F	AAAGCAGCCAAGAGGTTCC	005506.3	1488 – 1506	Primer-BLAST
	R	GTCTCCCGTTTCAACAAAGTC		1556 – 1536	
TFRC	F	GGCTACTTGGGCTATTGTAAAGG	003234.2	250 – 272	PrimerBank
	R	CAGTTTCTCCGACAACTTTCTCT		405 – 383	
TFR2	F	CGTGCGGAGACTCTGTGTT	003227.3	329 – 347	PrimerBank
	R	ATCCAGGTCAGGCTCATAGTT		387 – 367	
APPL1	F	ACTTGGGTACATGCAAGCTCA	012096.2	747 – 767	PrimerBank
	R	TCCCTGCGAACATTCTGAACG		863 – 843	
APPL2	F	AGCTGATCGCGCCTGGAACG	018171.3	1543 – 1562	Primer-BLAST
	R	GGGTTGGTACGCCTGCTCCCT		1636 – 1616	
EEA1	F	CCCAACTTGCTACTGAAATTGC	003566.3	497 – 518	PrimerBank
	R	TGTCAGACGTGTCACTTTTTGT		591 – 570	
RAB5A	F	AGACCCAACGGGCCAAATAC	004162.4	22 – 41	PrimerBank
	R	GCCCCAATGGTACTCTCTTGAA		164 – 143	
RABEP1	F	ATTAAGGCGATTGCCACAGTC	004703.4	486 – 506	PrimerBank
	R	TGGTGCTCATAGTCACGAACT		616 – 596	
RAB4A	F	GGGGCTCTCCTCGTCTATGAT	004578.2	470 – 490	PrimerBank
	R	AGCGCATTGTAGGTTTCTCGG		519 – 499	
RAB11	F	CAACAAGAAGCATCCAGGTTGA	004663.4	146 – 167	PrimerBank
	R	GCACCTACAGCTCCACGATAAT		260 – 239	
RAB25	F	TCGGCGAATCAGGTGTGGGGA	020387.2	279 – 299	Primer-BLAST
	R	ATGGTGGTGCGGCTGTCGTG		360 – 341	
RAB7A	F	GTGTTGCTGAAGGTTATCATCCT	004637.5	19 – 41	PrimerBank
	R	GCTCCTATTGTGGCTTTGTACTG		128 – 106	
RAB9	F	CCTCATTGCGCCCAGACGGG	004251.4	108 – 127	Primer-BLAST
	R	AGTGCAAGAGTGTCTCGCGGC		181 – 161	

2.5 Confocal microscopy

2.5.1 Immune fluorescence labelling

Non-specific antibody reactivity was limited by incubation of fixed cells with a 5 % (w/v) bovine serum albumin (BSA) in PBS solution for two hours at room temperature with slow agitation. Cells were then incubated with the appropriate concentration of primary antibody (Table 2.2) diluted in 5 % (w/v) BSA for two hours at room temperature followed by secondary antibody incubation for one hour at room temperature. Unbound antibody was removed by three PBS washes and coverslips mounted on 1 mm microscope slides with ProLong® Gold Antifade Reagent containing DAPI nuclear stain (Life Technologies).

2.5.2 Image acquisition and processing

Confocal microscopy was performed using a Zeiss LSM 710 META NLO laser-scanning microscope (University of South Australia, Australia) and associated Carl Zeiss Zen 2009 software. Laser lines of 370, 488, 543 and 633 nm were utilised for DAPI, Alexa Fluor® 488, Cy3 and Alexa Fluor® 633 fluorescence. Laser intensities were set between 5 % and 25 % depending on the fluorescent target. To eliminate signal crossover between fluorophores, emission fluorescence was filtered with a 20 nm gap between each channel. Each channel was captured by between-frame sequential scanning with a four-line average and a pixel-dwell of 3.15 µs. Pinhole diameters for each laser were set to 1 Airy unit. Cells were observed under a 1.4-apature 63× oil-immersion objective lens, with 2× optical zoom applied. Images were captured at a resolution of 1024 px^2 with a 16-bit greyscale depth. Based on image capture resolution of 1024 pixels and lens and zoom factor, each pixel was equivalent to 0.066 µM in dimension. At least four cells were randomly selected on each coverslip, and a representative image processed for the generation of figures. Images were

exported as grayscale 16-bit TIFF files, and any further processing performed using Adobe® Photoshop® CS5 (Adobe Systems Inc., San Jose, California, U.S.A).

2.5.3 Visualisation of intracellular trafficking and endocytosis

LysoTracker® positive vesicles were visualised by live-cell confocal microscopy to prevent leakage of LysoTracker®, which can occur from fixed cells. Cells were cultured as detailed in section 2.2.3.3 in hydrophobic chamber slides (DKSH Australia Pty Ltd., Victoria, Australia). Subsequently, LysoTracker® was diluted 1/200 in cell culture media and added to the cells, incubated for five minutes at 37 °C and the cells visualised as detailed in section 2.5.2.

Transferrin endocytosis was performed on cells cultured as detailed in section 2.2.3.3. At 48 hours, culture media was replaced with fresh culture media containing a 1/1000 dilution of transferrin-633 conjugate. Cells were incubated for a period of 5, 15 or 30 minutes, the culture media then aspirated and the cells washed briefly in DPBS, and fixed with 4 % PFA. Fixed cells were blocked for two hours with 5 % BSA before a 90-minute incubation with phalloidin-488 conjugate. Unbound antibody was removed by three DPBS washes and coverslips mounted on 1 mm microscope slides with ProLong® Gold Antifade Reagent containing DAPI nuclear stain (Life Technologies).

2.5.4 Co-fluorescence of transferrin and endosome markers.

Cells were cultured as detailed in section 2.2.3.3. At 48 hours, culture media was replaced with fresh culture media containing a 1/1000 dilution of transferrin-633 conjugate. Cells were incubated with transferrin-633 culture media for a period of 20 minutes. Subsequently, the culture media was aspirated, cells washed briefly with DPBS, and fixated

and permeabilised as detailed in section 2.2.3.3. Labelling with primary and secondary antibodies was performed as detailed in section 2.5.1.

2.6 Prostate cancer cohorts

The Taylor cohort consisted of patients treated by radical prostatectomy at the Memorial Sloan-Kettering Cancer Center (MSKCC) (Taylor et al. 2010), profiling 150 prostate cancer and 29 non-malignant tissues which was performed using Affymetrix Human Exon 1.0 ST arrays. Data was obtained from the cBio Cancer Genomics Pathway Portal[6]. The Chandran cohort (Chandran et al. 2007) was composed of 18 normal tissues, 65 primary, 25 metastatic and 63 normal adjacent non-malignant tissues. Expression profiling of these tissues was carried out using Affymetrix U95Av2 human gene arrays. Data was retrieved from NCBI GEO (accession number GSE6919). A cohort by Tomlins (Tomlins et al. 2007), was retrieved from NCBI GEO (accession number GSE6099) and was comprised of 32 primary, 20 metastatic, 13 prostatic intraepithelial neoplasia (PIN), and 27 normal tissue samples. Analysis of samples was previously performed using a Chinnaiyan Human 20K Hs6 array (Tomlins et al. 2007). An additional cohort from MSKCC, the Glinsky cohort (Glinsky et al. 2004) was composed of 79 malignant prostate tissues with clinical follow-up data to 106 months that was used to assess for biochemical recurrence of prostate cancer. This comprised 29 patients with biochemical recurrence as defined by a PSA concentration ≥ 0.2 ng/mL and 50 patients with no progression.

Statistical analysis between normal and prostate cancer tissue was performed using a two-tailed unpaired t test with Welch's correction. To evaluate differences in expression between multiple groups in the Chandran and Tomlins cohort, a Kruskal-Wallis with

[6] cbio.mskcc.org/cancergenomics-dataportal (accessed June 2012)

Dunn's multiple comparison test was performed. Data from the Glinsky cohort was analysed using a two-tailed unpaired t test with Welch's correction to determine differences between Gleason grade 3 and 4 groups. *K*-means clustering was implemented using Stata/SE v11.2 (StataCorp LP, Texas, U.S.A) to determine high and low gene expression groups and evaluated using Kaplan-Meier survival curves with Gehan-Breslow-Wilcoxon test to determine difference between the curves. All statistical tests were performed using GraphPad Prism 5.03 (GraphPad Software Inc., California, U.S.A). Significant results showed greater than 95 % confidence ($P \leq 0.05$).

Chapter 3

Evaluating cell lines and lysosomal proteins for prostate cancer biomarker discovery

3.1 Introduction

Current methods for prostate cancer detection are limited for specificity and sensitivity; and cannot discriminate patients who are at higher risk of progressive disease or mortality from those who have a more favourable prognosis. For example, the primary marker used in prostate cancer diagnosis, prostate specific antigen (PSA)/human kallikrein 3 (hK3), does not adequately discriminate between cancer or benign pathologies (De Nunzio et al. 2011; Tarhan et al. 2005), resulting in false-negative (Lucia et al. 2008; Thompson, IM et al. 2004) or false-positive diagnoses (Grubb et al. 2008; Schroder, FH et al. 2009). Moreover, PSA cannot distinguish between aggressive or more slow-growing cancers at the time of diagnosis, and this results in over-treatment (Sandhu & Andriole 2012; Schroder, FH et al. 2009). Consequently, there have been recent recommendations to abandon PSA screening in the USA (Moyer 2012). There is therefore a significant need for new or additional prostate cancer biomarkers that enable effective diagnosis and prognosis.

The sensitivity and specificity of other secondary prostate cancer biomarkers varies considerably. These biomarkers include lysosomal proteinases, such as cathepsins B and D, which have been detected in secretions from malignant cells derived from a variety of tissues (Recklies et al. 1980; Sinha et al. 2001; Sloane, Bonnie F. et al. 1982; Troy et al. 2004). The amount of β-human chorionic gonadotrophin (β-hCG) protein is increased in the serum and urine of patients with prostate cancer, and this biomarker is associated with low-grade disease (Daja et al. 2000), however β-hCG is unable to distinguish patients at risk of mortality, and elevated amounts in serum or urine are only observed in a small proportion (4–10 %) of patients (Otite et al. 2006). The carbohydrate antigen biomarkers CA 15-3, CA 19-9, C-reactive protein and carcinoma embryonic antigen, whilst

maintaining some prognostic capabilities, provide minimal diagnostic value due to their low specificity and the need for a prostate biopsy for this analysis (Falsaperla et al. 2003; Sella et al. 2000); and are consequently not being investigated for use in prostate cancer diagnosis (Sturgeon et al. 2008). Thus, detection of serum PSA concentration combined with digital rectal examination remains the recommended methods of diagnosis, prognosis and disease monitoring[7]. There is therefore an urgent need for a more specific and accurate detection method for prostate cancer, to allow early diagnosis and selection of the most appropriate therapeutic interventions. Early detection significantly reduces mortality from prostate cancer (Schroder, FH et al. 2012), making accurate diagnostic and prognostic methods an important objective.

Cell lines have been heavily utilised for prostate cancer biomarker discovery, however, there have been significant problems with these studies, in terms of the number and type of prostate cell lines examined. Studies involving multiple non-malignant control cell lines have been limited, despite the number of prostate cell lines available (see for example: Colella, Rita, Goodwyn & Gopal 2002; Gao et al. 2012; Moro, L. et al. 2007; O'Connell et al. 2012; Sardana et al. 2008; Sardana, Marshall & Diamandis 2007). The large number of studies characterising individual prostate cell lines may have actually been detrimental to effective biomarker discovery; as these candidate markers may not be commonly expressed in other prostate cancer cells or have the capacity to discriminate between non-malignant and cancer cells. Meta-analysis of the literature has resulted in the formulation of a database[8], compiling protein and gene expression data for PSA, androgen receptor,

[7] aacc.org/members/nacb/LMPG/OnlineGuide/PublishedGuidelines/major/Pages/toc.aspx (accessed May 2013)
[8] capcelllines.ca/downloads/CaPCellLines_database.xls (accessed December 2012)

kallikrein 2, and a limited number of other markers. There have been some marker profiles performed on multiple cell lines, for example analysing androgen receptor mutations, amount of androgen receptor protein, androgen response and defining p53 or TP53 mutations (Sobel & Sadar 2005b, 2005a; van Bokhoven et al. 2003). These marker profiles have also been assessed in relation to growth and invasive capacity (Webber, Bello & Quader 1996, 1997a, 1997b). However, a systematic study to identify novel biomarkers using multiple non-malignant and prostate cancer cell lines has not been performed.

Lysosomal enzymes have been historically implicated in prostate cancer biology and pathogenesis, and a limited number of lysosomal proteins have been evaluated as biomarkers. In prostate cancer epithelial cells, increases in the number and size of lysosomes have been reported compared to cells of non-malignant prostate tissue (Aughey, Scott & McLaughlin 1974) and increased lysosomal acid phosphatase staining and increased amounts of acid phosphatase in cancer tissue and in serum from cancer patients have been demonstrated compared to normal prostate tissue (Fischer et al. 1979). Lysosomal acid phosphatase (Henneberry, Engel & Grayhack 1979) and various glycosidases including acid ceramidase have altered gene expression in a range of cancers, including prostate cancer (Seelan et al. 2000; Sinowatz et al. 1978). Acid ceramidase can be upregulated in prostate cancer tissues compared with normal prostate tissue, and increased quantities of this enzyme have been implicated in a poor response to cancer treatment (Liu, X et al. 2009; Mahdy et al. 2009). Increased amounts of cathepsin B protein in prostatic intraepithelial neoplasia and prostate cancer have also been observed, together with altered enzyme localisation to the plasma membrane of these cancer cells (Sinha et al. 2001; Sinha et al. 1995b), suggesting that altered lysosomal biogenesis may be involved in cancer onset and progression. While increased amounts of lysosomal hydrolases and

changes in lysosomal distribution have been implicated in prostate cancer biology, a detailed study of lysosomal biogenesis has not been performed in prostate cancer.

This study has evaluated a number of candidate lysosomal hydrolases and a set of existing prostate cancer biomarkers across a panel of non-malignant and prostate cancer cell lines, to determine if there is consistent alteration for protein amount between prostate cancer and non-malignant control cell lines.

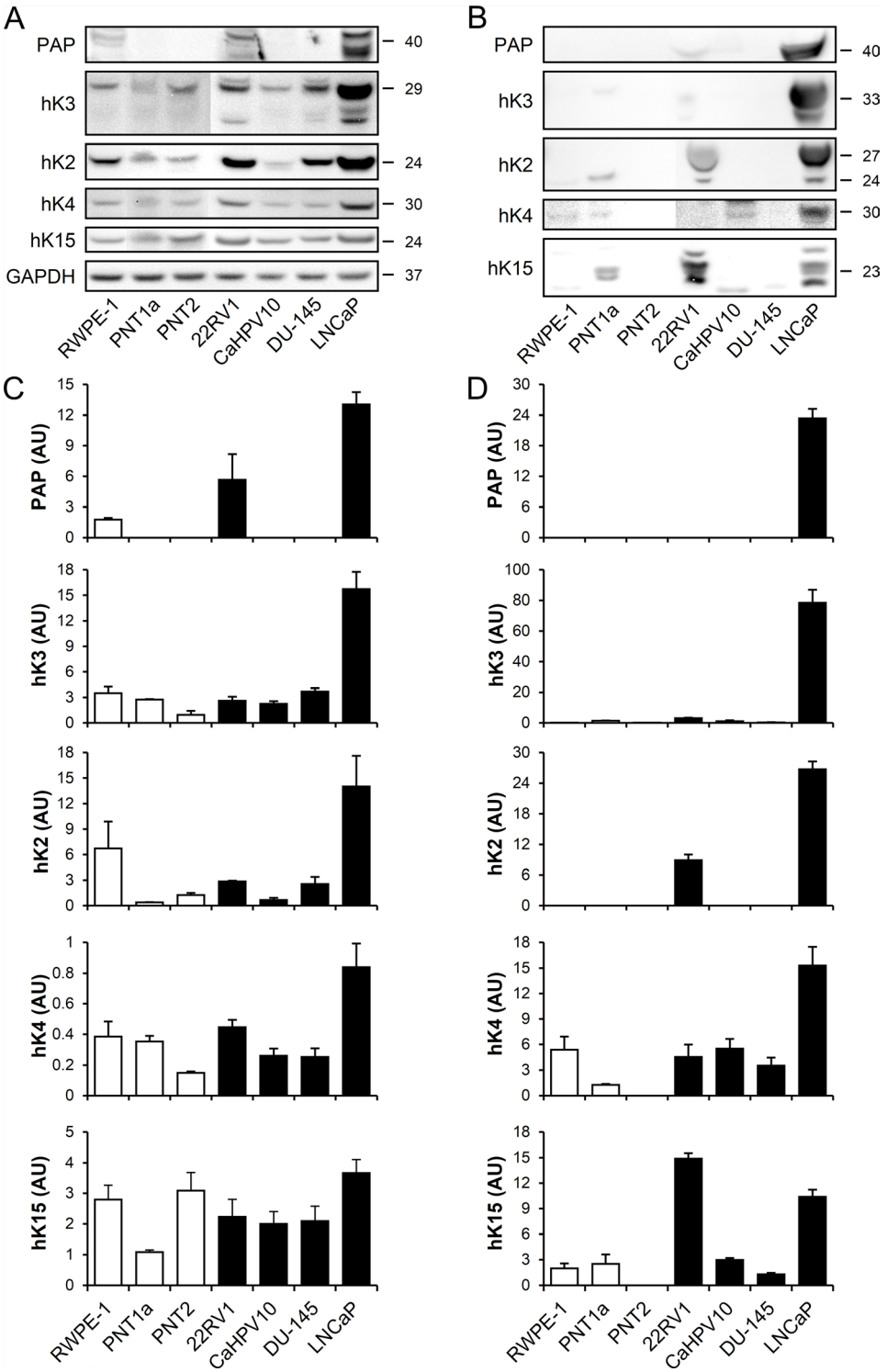
3.2 Results

3.2.1 Detection of current prostate cancer biomarkers in prostate cancer cell extracts and culture media.

Western blot analysis was used to define the amount of prostatic acid phosphatase (PAP), prostate-specific antigen (hK3) and other kallikreins (hK2, hK4 and hK15) in cell lysates and culture media from the non-malignant prostate cell lines RWPE-1, PNT1a and PNT2 and the prostate cancer cell lines 22RV1, LNCaP, CaHPV10 and DU-145 (Figure 3.1). While the intracellular amount of PAP was four to eight-fold higher in the prostate cancer cell lines 22RV1 and LNCaP, when compared to the non-malignant control cell lines, little or no protein was detected in the CaHPV10 and DU-145 prostate cancer cell lines (Figure 3.1 A & C). Similarly, hK2, hK3 and hK4 showed increased amounts of intracellular protein of between two to five-fold in LNCaP when compared to the non-malignant control cell lines, but minimal amounts were detected in the other prostate cancer cell lines (Figure 3.1 A & C). The amount of intracellular hK15 was similar for all of the prostate cancer cell lines, as well as the non-malignant control cell lines RWPE-1 and PNT2, albeit with an approximately three-fold decrease in the amount detected in PNT1a (Figure 3.1 A & C). PAP, hK3, hK2, and hK4 were secreted from LNCaP cells and the amount was increased when compared to the other prostate cancer and non-malignant control cell lines (Figure 3.1 B & D). Notably, the secretion of hK15 from 22RV1 and LNCaP displayed an approximate four-fold elevation when compared to the other prostate cancer and non-malignant control cell lines (Figure 3.1 B & D). These findings indicated variable protein content, and PAP/kallikrein secretion for the prostate cancer cell lines and an inability of these markers to distinguish between a number of prostate cancer and non-malignant control cell lines.

The detection of hK2, hK4 and hK15 in cell extracts revealed single molecular forms that could not be used to discriminate between non-malignant control and prostate cancer cell lines (Figure 3.1 A & C). Prostatic acid phosphatase was variably processed in the 22RV1 and LNCaP prostate cancer cell lines (40 and 38 kDa molecular forms) compared to the non-malignant cell line RWPE-1 (38 kDa form), but was absent from the other cell lines (Figure 3.1 A & C). Similarly, two molecular forms of hK3 were detected in 22RV1 and LNCaP prostate cancer cell lines (29 and 25 kDa), while the other control and cancer cell lines only expressed the 29 kDa molecular form (Figure 3.1 A & C). The detection of secreted hK2 and hK15 revealed molecular forms (27 kDa for hK2; 21 kDa and 25 kDa for hK15) that were similar to PNT1a but these molecular forms were not detected in the other non-malignant control and cancer cell lines (Figure 3.1 A & C). This indicated variable proteolytic processing in some prostate cancer cell lines when compared to the non-malignant control cell lines.

The secretion of kallikreins appeared to correlate with the androgen-receptor status of prostate cancer cell lines and the amounts secreted generally appeared to be independent of the amounts detected in the cell extracts (Figure 3.1 B & D). An increased secretion of hK3, hK2 and hK15 was observed from 22RV1 and LNCaP prostate cancer cell lines (Figure 3.1 B & D). However, the amount of hK2 was elevated in cell extracts from the RWPE-1 non-malignant cells when compared to 22RV1; while there was increased secretion of this marker from the 22RV1 cancer cell line compared to the RWPE-1 non-malignant control cell line (Figure 3.1 B & D). Elevated amounts of kallikreins are expected in cell lines that are responsive to androgen, or possessing activated androgen receptor, thereby activating genes containing androgen-response elements (Luke & Coffey 1994; Murtha, Tindall & Young 1993).


A
PAP
hK3
hK2
hK4
hK15
GAPDH
40
29
24
30
24
37
RWPE-1
PNT1a
PNT2
22RV1
CaHPV10
DU-145
LNCaP
B
PAP
hK3
hK2
hK4
hK15
40
33
27
24
30
23
C
PAP (AU)
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hK4 (AU)
hK15 (AU)
D
PAP (AU)
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hK2 (AU)
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hK15 (AU)

Figure 3.1 – Detection and quantification of known prostate cancer biomarkers in non-malignant and prostate cancer cell lines.

Western blots of biomarkers/GAPDH in cell extracts **(A**; 50 μg of whole cell lysate**)** and culture media (**B**; 3 mL culture media, collected after 48 hours incubation with confluent cells) from the non-malignant control cell lines RWPE-1, PNT1a and PNT2, and prostate cancer cell lines 22RV1, CaHPV10, DU-145 and LNCaP. Western blots are representative of triplicate experiments (n = 3). The amount of each intracellular (C) or secreted (D) protein was quantified from the Western blots by densitometry (arbitrary units) relative to GAPDH (endogenous control). Non-malignant cell lines are depicted by white bars and prostate cancer cell lines by black bars and results represent the mean ± SE (n = 3).

3.2.2 Detection of lysosomal proteins in non-malignant control and prostate cancer cell extracts and culture media.

Western blot analysis was used to define the amount of different lysosomal proteins in cell lysates and culture media from non-malignant control and prostate cancer cell lines (Figure 3.2 A & B; Figure 3.3 A & B). The amounts of intracellular α-glucosidase (α-Gluc), β-glucosidase (β-Gluc), α-galactosidase A (α-Gal A), LAMP-1 and cathepsin D (26 kDa) was variable between the cell lines and could not differentiate non-malignant controls from the prostate cancer cell lines (Figure 3.2 A; Figure 3.3 A). The amount of pro-cathepsin D in cell extracts was similar for non-malignant control, CaHPV10 and DU-145 cancer cell lines, however there was no detectable protein in the cell extract of 22RV1 and LNCaP prostate cancer cell lines (Figure 3.2 A; Figure 3.3 A). The secretion of β-glucosidase, α-galactosidase, pro-cathepsin B and D was elevated in RWPE-1 when compared to the other non-malignant cell lines PNT1a and PNT2 (Figure 3.2 B; Figure 3.3 B). The amount of α-galactosidase secreted from RWPE-1 was similar to that from the CaHPV10 prostate cancer cell line (Figure 3.2 B; Figure 3.3 B). The amount of LAMP-1 and cathepsin D (26 kDa) secretion was similar for each of the non-malignant control and prostate cancer cell lines (Figure 3.2 B; Figure 3.3 B). The 22RV1 and DU-145 prostate cancer cell lines had elevated secretion of α-glucosidase and β-glucosidase when compared to the other prostate cancer and non-malignant control cell lines (Figure 3.2 B; Figure 3.3 B). Limited amounts of cathepsin B (22 kDa) was detected in the 22RV1 prostate cancer cell line when compared to the other prostate cancer and non-malignant control cell lines (Figure 3.2 A & B; Figure 3.3 A & B). The amount of pro-cathepsin B was elevated in LNCaP when compared to the other prostate cancer cell lines (Figure 3.2 A; Figure 3.3 A). RWPE-1 had an increased amount of pro-cathepsin B when compared to the PNT1a and PNT2 non-

malignant control cell lines (Figure 3.2 A & B; Figure 3.3 A & B). The secretion of cathepsin B (22 kDa) was higher for CaHPV10, DU-145 and LNCaP prostate cancer cell lines compared to the non-malignant control cell lines and the 22RV1 prostate cancer cell line (Figure 3.2 B; Figure 3.3 B). Minimal amounts of acid ceramidase were detected in the non-malignant control cell lines and the prostate cancer cell lines 22RV1, CaHPV10 and DU-145 (Figure 3.2 A; Figure 3.3 A). In contrast, large amounts of acid ceramidase were detected in cell extracts from the LNCaP cancer cell line (Figure 3.2 A; Figure 3.3 A). Acid ceramidase (10-15 kDa) was not secreted from the non-malignant control cell lines, but elevated amounts were secreted from the 22RV1, DU-145 and LNCaP prostate cancer cell lines when compared to CaHPV10 and the non-malignant control cell lines (Figure 3.2 B; Figure 3.3 B). These findings indicated variable intracellular content and secretion of lysosomal proteins for non-malignant control and prostate cancer cell lines, with a potential for cathepsin B and acid ceramidase to distinguish between non-malignant control and prostate cancer cell lines. The secretion of acid ceramidase and cathepsin B may therefore afford some capacity to distinguish between non-malignant and cancer cell lines.

Multiple molecular forms of α-glucosidase (α-Gluc) were detected in the cell extracts of non-malignant and prostate cancer cell lines. A 110 kDa molecular form of α-glucosidase was detected in 22RV1 and LNCaP cancer cell lines, but only minimal amounts were detected in the other prostate cancer and non-malignant control cell lines (Figure 3.2 A; Figure 3.3 A). The molecular weight of β-glucosidase (β-Gluc) and LAMP-1 varied between prostate cancer and non-malignant cell lines and was unable to discriminate between these types of cell lines (Figure 3.2 A). Two molecular forms of α-galactosidase A (α-Gal A; 42 and 43 kDa) were present in both CaHPV10 and RWPE-1 cell lines, but not

in other prostate cancer or non-malignant cell lines (Figure 3.2 A). Both CaHPV10 and RWPE-1 displayed low amounts of the 70 kDa molecular form of α-glucosidase when compared to the other non-malignant and prostate cancer cell lines (Figure 3.2 A). Interestingly, both RWPE-1 and CaHPV10 cell lines displayed similar protein processing of α-glucosidase and α-galactosidase A, and a similarly increased molecular weight of β-glucosidase and elevated secretion amounts of α-galactosidase A, which differed from the other non-malignant and prostate cancer cell lines; and this correlated with the different technique used to immortalise these two cell lines (Figure 3.2 A & B). Two molecular forms of pro-cathepsin B (37 and 39 kDa) were detected in the cell extract of CaHPV10, DU-145 and LNCaP prostate cancer and RWPE-1 non-malignant control cell lines (Figure 3.2 A). A 60 kDa molecular form of β-glucosidase was secreted from RWPE-1 and CaHPV10 cells, which was more diffuse from the other cell lines and suggested variable glycosylation (Figure 3.2 B). The molecular weight of the secreted form of α-galactosidase A was lower in the prostate cancer cell lines 22RV1, DU-145 and LNCaP when compared to the non-malignant control cell lines and CaHPV10 prostate cancer cell line (Figure 3.2 B). The variable processing and increased secretion of some lysosomal proteins may have some capacity to distinguish between prostate cancer and non-malignant cell lines.

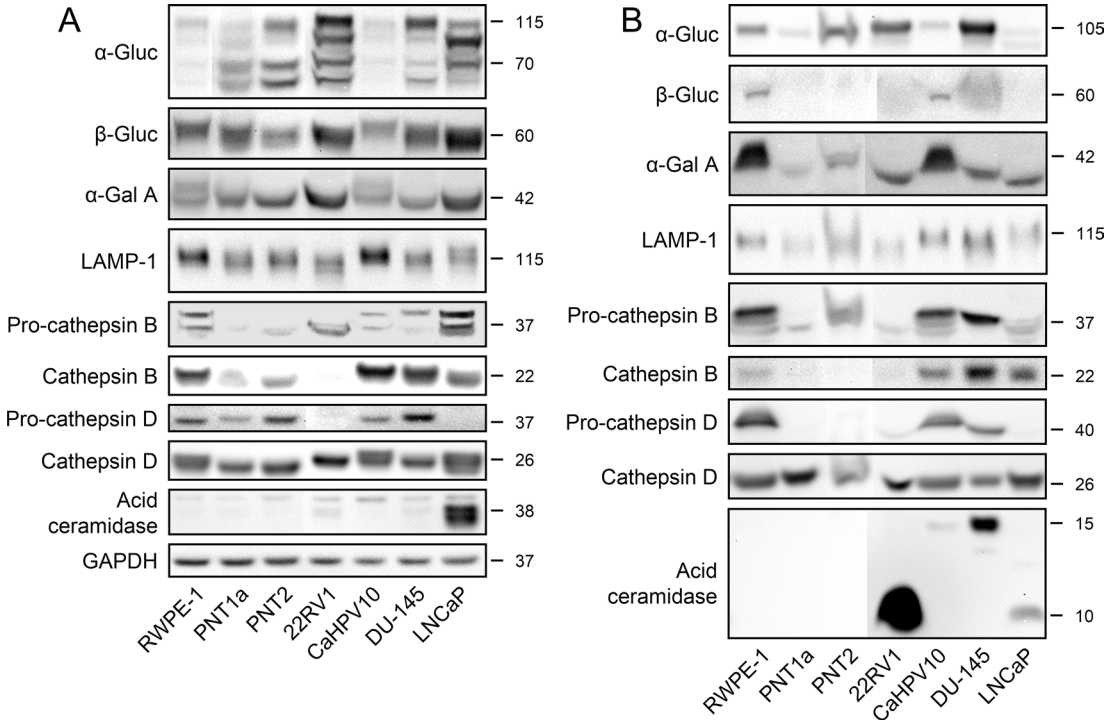


Figure 3.2 – Detection of lysosomal proteins in non-malignant and prostate cancer cell lines.

Western blots of lysosomal proteins/GAPDH in cell extracts **(A;** 50 µg of whole cell lysate**)** and culture media (**B**; 3 mL culture media, collected after 48 hours incubation with confluent cells) from the non-malignant control cell lines RWPE-1, PNT1a and PNT2, and prostate cancer cell lines 22RV1, CaHPV10, DU-145 and LNCaP. Western blots are representative of triplicate experiments.

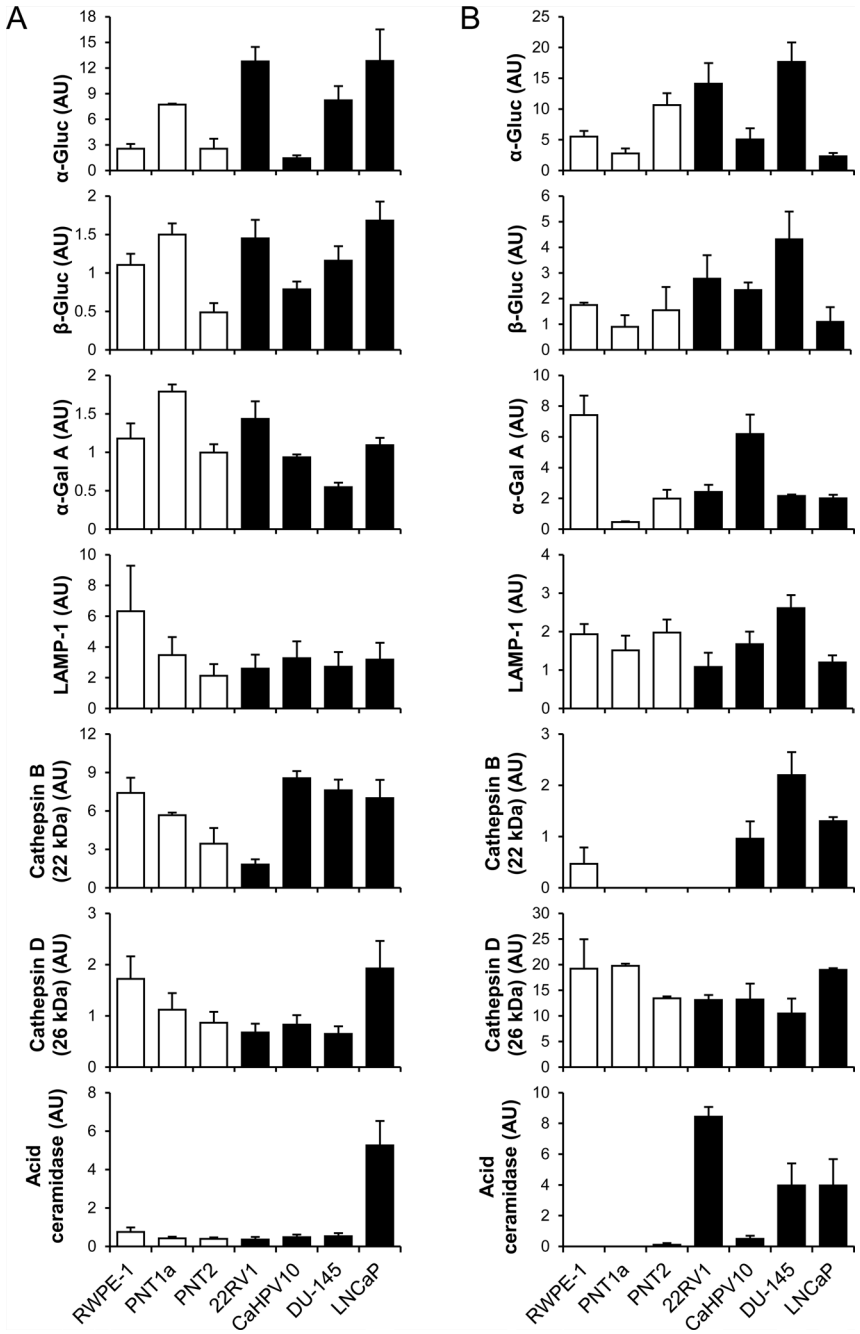


Figure 3.3 – Quantification of lysosomal proteins in non-malignant and prostate cancer cell lines.

The amount of intracellular protein relative to GAPDH endogenous control (**A**) and secreted lysosomal protein obtained from 3 mL conditioned culture media (**B**) was quantified from the Western blots (Figure 3.2) by densitometry (arbitrary units). The amount of GAPDH was determined for each individual Western blot. Non-malignant cell lines are depicted by white bars and prostate cancer cell lines by black bars and results represent the mean ± SE (n = 3).

3.3 Discussion

Prostate cancer cell lines potentially provide a valuable model for the discovery and evaluation of biomarkers. A major advantage for culture systems is that they provide a source of cancer derived cells that is homogenous and can be reproducibly generated *in vitro*. However, previous studies of biomarkers using prostate cancer cell lines have been restricted by the limited use of non-malignant control cell lines, despite the number of prostate cell lines available. Most studies have also only tended to look at one or two prostate cancer cell lines in isolation, instead of a panel of cell lines. A major disadvantage of *in vitro* cell culture systems is that they do not reproduce the physiological conditions and tissue microenvironment encountered *in vivo,* but rather rely on culture media that may independently alter biomarker expression (Cukierman et al. 2001). However, *in vitro* systems may still provide the advantage of relative simplicity and consistency, particular when analysing low abundance proteins that may be masked by albumins, immunoglobulins and other abundant proteins in biofluids or tissue sources. It is therefore important to appreciate the potential limitations of *in vitro* studies when analysing biomarkers in prostate cell lines and to perform follow up studies that establish the *in vivo* validity of potential biomarkers, to demonstrate that they are not an artefact of *in vitro* culture. We propose that an essential first step in an *in vitro* prostate cancer-biomarker discovery process must involve the use of multiple non-malignant and prostate cancer cell lines to establish biomarker reproducibility and potential for discrimination.

The primary marker for prostate cancer is currently PSA (hK3), but there are significant problems with false negatives, the discrimination of benign pathologies and over-diagnosis (Bunting 1995; De Nunzio et al. 2011; Nadler et al. 1995; Schroder, FH et al. 2009; Tarhan et al. 2005; Thompson, IM et al. 2004). These limitations for PSA were evident here in the

cell line analysis with high amounts of hK3 detected in LNCaP and significant secretion of this biomarker from this, but not the other prostate cancer cell lines examined. Kallikrein expression is regulated by androgen-stimulated androgen receptor (Heinlein & Chang 2004; Luke & Coffey 1994), through androgen response elements present on kallikrein genes (Murtha, Tindall & Young 1993; Stephenson et al. 1999; Yousef et al. 2002). The increased amount of hK3 and other kallikreins in the LNCaP cell line has previously been well described and reflects the androgen responsiveness of these cells (Young, Andrews & Tindall 1995). A similar, but not identical pattern of kallikrein detection was observed in 22RV1, although there were marked differences in the secretion profile for different kallikreins, which may reflect the altered androgen receptor variants present in this line. The variable expression and secretion of kallikreins in non-malignant and cancer cell lines was observed in prostate cancer patients that are frequently androgen receptor-positive and responsive to androgens (Basu & Tindall 2010; Qiu, Leuschner & Braun 2008). The secretion of acid phosphatase was also variable in prostate cancer cell lines, and there have been reports of reduced amounts of this biomarker associated with metastatic prostate cancer compared with normal tissue and acid phosphatase and has been implicated in the androgen responsiveness of prostate cancer (Veeramani et al. 2005). The combination of multiple non-malignant and prostate cancer cell lines may to some extent indicate the variable phenotype of prostate cancer and thereby provide a model for additional biomarker discovery relating to cancer sub-types.

Lysosomal proteins have been implicated in prostate cancer biology and pathogenesis, through increased cellular content and secretion of lysosomal proteins (Aughey, Scott & McLaughlin 1974; Henneberry, Engel & Grayhack 1979); and this may affect proliferation, angiogenesis and metastasis (Nomura & Katunuma 2005; Steffan, J. J. & Cardelli 2010).

There is conflicting evidence for altered amounts of cathepsin D in prostate cancer (Nicotra et al. 2010). While there was no difference in the secretion of mature cathepsin D from prostate cancer compared to non-malignant cell lines, there was increased secretion of procathepsin D. Previous studies have described the secretion of procathepsin D and defective separation from the mannose-6-phosphate receptor in the endosomal compartments (Isidoro, Baccino & Hasilik 1997), consequently diverting the protein to the secretory pathway and thus avoiding maturation. Altered pro-cathepsin D trafficking towards the secretory pathway may arise from the deletion of the signal peptide, as has been reported for cathepsin H in prostate cancer (Waghray et al. 2002), or through alternate trafficking by the sortilin receptor (Canuel et al. 2008; Coutinho, Prata & Alves 2012b; Rijnboutt et al. 1991). Post-translational modifications and altered glycosylation of pro-cathepsin D may also affect the trafficking of this protein, as reported in breast cancer cells (Capony et al. 1989). The differential processing of lysosomal proteins that was observed in prostate cancer cell lines in this study may affect cell proliferation (Moro, Loredana et al. 2004) and receptor activation/processing (Grimsey, Soto & Trejo 2011; Shi et al. 2004). These modifications that affect trafficking or regulation can also alter protein activity and stability (Michaeli et al. 2012) and this may contribute to the malignant phenotype. The analysis of lysosomal protein processing may offer alternative biomarkers for prostate cancer, but further investigation of protein processing and its downstream affects are required.

While cell lines offer some advantages for the analysis of prostate cancer biology, the conditions required to establish stable cell lines might induce abnormal phenotypes. Viral transformation is required for the continued proliferation of epithelial prostate cells *in vitro*. PNT1a and PNT2 non-malignant cell lines have been immortalised with SV40, and

the non-malignant RWPE-1 and prostate cancer cell line CaHPV10 have been immortalised with HPV-18 (Bello et al. 1997; Weijerman et al. 1994). As a result of HPV transfection, RWPE-1 expresses E6 and E7 oncoproteins (CaHPV10 expresses only E6). Deactivation of E2F transcription factor by the action of E6 and E7 on retinoblastoma protein (pRb) enables cell cycle progression to continue unimpeded. E7 and E6 oncoproteins further stabilise and induce the accumulation of p53 (Jones & Munger 1997; Seavey et al. 1999), which may also affect lysosomal stability (Yuan et al. 2002). Thus, the amount and processing of protein observed for RWPE-1 and CaHPV10 cell lines may be a consequence of their immortalisation by HPV-18, inducing aberrant amplification of target genes and altered protein abundance and processing. These effects are not maintained in the SV40-transfected cells PNT1a and PNT2 that exclusively express TAg (Tiemann & Deppert 1994), and lack a p53-stabilising agent. Thus, the PNT1a and PNT2 non-malignant cell lines may be more representative of normal prostate epithelial cells than the cell line RWPE-1. It is therefore concerning that many past studies have used only the RWPE-1 non-malignant cell line as a control for prostate cancer cell line studies. The use of multiple non-malignant cell lines such as PNT1a and PNT2 could improve the reliability of the data and conclusions derived from *in vitro* studies.

Elevated secretion of acid ceramidase and mature cathepsin B from prostate cancer cell lines suggested that the endosomal network might be altered in prostate cancer, providing an alternative set of potential biomarkers. Cathepsin B has been reported to have mainly an endosomal intracellular location (Authier, Kouach & Briand 2005; Authier et al. 1999), when compared to the lysosomal location of another candidate prostate cancer biomarker, cathepsin D (Zaidi et al. 2008). Acid ceramidase is processed to a mature form in acidic endosomal compartments (Ferlinz et al. 2001) and has a specific functional role in

multivesicular endosomes (Trajkovic et al. 2008). Acid ceramidase is upregulated in prostate cancer tissues and an increased abundance of this enzyme has been implicated in a poor response to cancer treatment (Seelan et al. 2000). The intracellular trafficking of acid ceramidase is mannose-6-phosphate receptor-mediated, but altered glycosylation can divert this enzyme into secretory vesicles (Zeidan et al. 2008). Here, the α-subunit was detected in prostate cancer cells, consistent with traffic via the mannose-6-phosphate receptor to endosomes, but the secretion of this subunit implied that acid ceramidase might have been released from these compartments. While there was some evidence of alterations in lysosomal compartments and their constituent hydrolases in prostate cancer cells, these findings suggested that endosome biogenesis might be more consistently altered than that of lysosomes, providing a new avenue for biomarker discovery.

In conclusion, through the evaluation of non-malignant and prostate cancer cell lines we have determined that endosome-constituents may be a source of candidate biomarkers for prostate cancer, whilst lysosomal hydrolases and existing prostate cancer biomarkers are not able to differentiate between non-malignant and cancer cell lines. Comparison between multiple non-malignant and prostate cancer cell lines suggests that some HPV-transformed cells may exhibit characteristics that are not representative of non-malignant or prostate cancer cells.

Chapter 4

Altered endosome biogenesis in prostate cancer

4.1 Introduction

Prostate cancer is the most common form of cancer in males from developed countries, and the incidence of this disease is predicted to double globally by 2030 (WCRF prostate cancer statistics[9]). In Australia and New Zealand, the incidence of prostate cancer is higher than other Western countries and is 25 times higher than South-Central Asia. In 2009, more than nineteen thousand Australian men were diagnosed with prostate cancer, and there were nearly three thousand deaths; making this disease one of the largest causes of cancer-related deaths.

The prostate-specific antigen test is currently used for prostate cancer diagnosis, however, this assay suffers particularly from a high percentage of false-positive results (Ilic et al. 2007; Ilic et al. 2011) and recently there have been recommendations to abandon this procedure, particularly in older men (Moyer 2012). The digital rectal examination is an alternative procedure to check the prostate for abnormalities, but this test is limited by the inability to assess the whole gland and to some degree the size of the tumour. Early detection of prostate cancer is critical as it enables more effective treatment, with reduced mortality. Understanding the cell biology of prostate cancer is important in order to develop new approaches for the early diagnosis, prognosis and treatment of prostate cancer.

Cumulative evidence from patient studies supports the concept that the process of lysosomal biogenesis is altered in prostate cancer. Lysosomal acid phosphatase (Henneberry, Engel & Grayhack 1979) and various glycosidases, including β-glucosidase, acid ceramidase and β-N-Acetylglucosaminidase have been shown to have altered

[9] http://globocan.iarc.fr (accessed March 2013)

expression in prostate cancer (Sinowatz et al. 1978). Moreover, C-reactive protein, which is associated with inflammatory immune responses, has also been found to be elevated in the circulatory system of prostate cancer patients (Kato et al. 2006). Acid ceramidase is upregulated in prostate cancer tissues and the overexpression of this enzyme has been implicated in a poor response to cancer treatment (Ferlinz et al. 2001). *In vitro* studies have also investigated some lysosomal proteins in prostate cancer cells. Increased amounts of acid ceramidase have been reported in prostate cancer cells when compared to benign prostatic cells (Seelan et al. 2000). Increased amounts of membrane associated acid phosphatase have been reported in PC3 and LNCaP cell lines, but with a reduced level of soluble acid phosphatase secretion (Quintero et al. 2007). The cathepsins (B, D, L, and H) have also been reported to be elevated in some prostate cancer cell lines (Chauhan, Goldstein & Gottesman 1991; Friedrich et al. 1999; Waghray et al. 2002). Despite these findings, there has not been a systematic study of endosome-lysosome biogenesis in prostate cancer.

Endosome-lysosome biogenesis involves the synthesis and organisation of structural elements of the endosome-lysosome system to form an integrated set of functional organelles. There are two main pathways to the lysosome; from the biosynthetic compartment (endoplasmic reticulum and Golgi apparatus) via specific vesicular traffic to the endosome network (late endosomes and multivesicular bodies); and from the cell surface through early endosomes and recycling endosomes, which then interact with other compartments of the endosome-lysosome system. In both cases the formation and movement of these dynamic vesicular compartments is controlled by specific targeting signals and trafficking machinery. This vesicular machinery can be used to define individual compartments; for example, the small GTPase Rab5 on early endosomes, the small

GTPase Rab7 on late endosomes and the integral lysosomal membrane protein LAMP-1 that is enriched in lysosomal membranes.

We have defined the gene expression, as well as protein amount and intracellular distribution for a panel of endosome and lysosome proteins in non-malignant control and prostate cancer cell lines. The gene expression and amount of LAMP-1 protein was similar in prostate cancer and non-malignant control cell lines; however, there was increased expression of the integral membrane protein LIMP-2 and evidence of altered endosome morphology. Overexpression of LIMP-2 has been shown to cause an enlargement of early and late endosomes (Kuronita et al. 2002); leading us to focus this prostate cancer cell line study on endosome vesicular machinery and the distribution of endosome subsets.

4.2 Results

4.2.1 Increased endosome related gene and protein expression in prostate cancer cells.

The expression of endosome and lysosome related genes was quantified by qRT-PCR in non-malignant control and prostate cancer cells and normalised to the expression of *GAPDH* mRNA. The amounts of *APPL1*, *APPL2*, *EEA1*, *RAB5A*, *RAB4A* and *LIMP2* mRNA were significantly increased in prostate cancer when compared to non-malignant control cell lines ($P \leq 0.05$; Figure 4.1). In each case there was an approximately 2-3 fold increase in mRNA expression. There was not a significant difference in the amount of either *RAB7A* or *LAMP1* mRNA detected in prostate cancer cells compared to non-malignant controls. Western analysis demonstrated significant increases in the amount of Appl1, Appl2, EEA1, Rab4 and LIMP-2 protein in extracts from prostate cancer cells when compared to non-malignant control cells ($P \leq 0.05$; Figure 4.2). Moreover, for both LIMP-2 and Rab4 the increase was approximately 2-4 fold for cancer when compared to non-malignant control cells (Figure 4.2). There was not a significant difference in the amount of Rab5A, Rab7 and LAMP-1 protein detected in non-malignant control compared to prostate cancer cells (Figure 4.2).

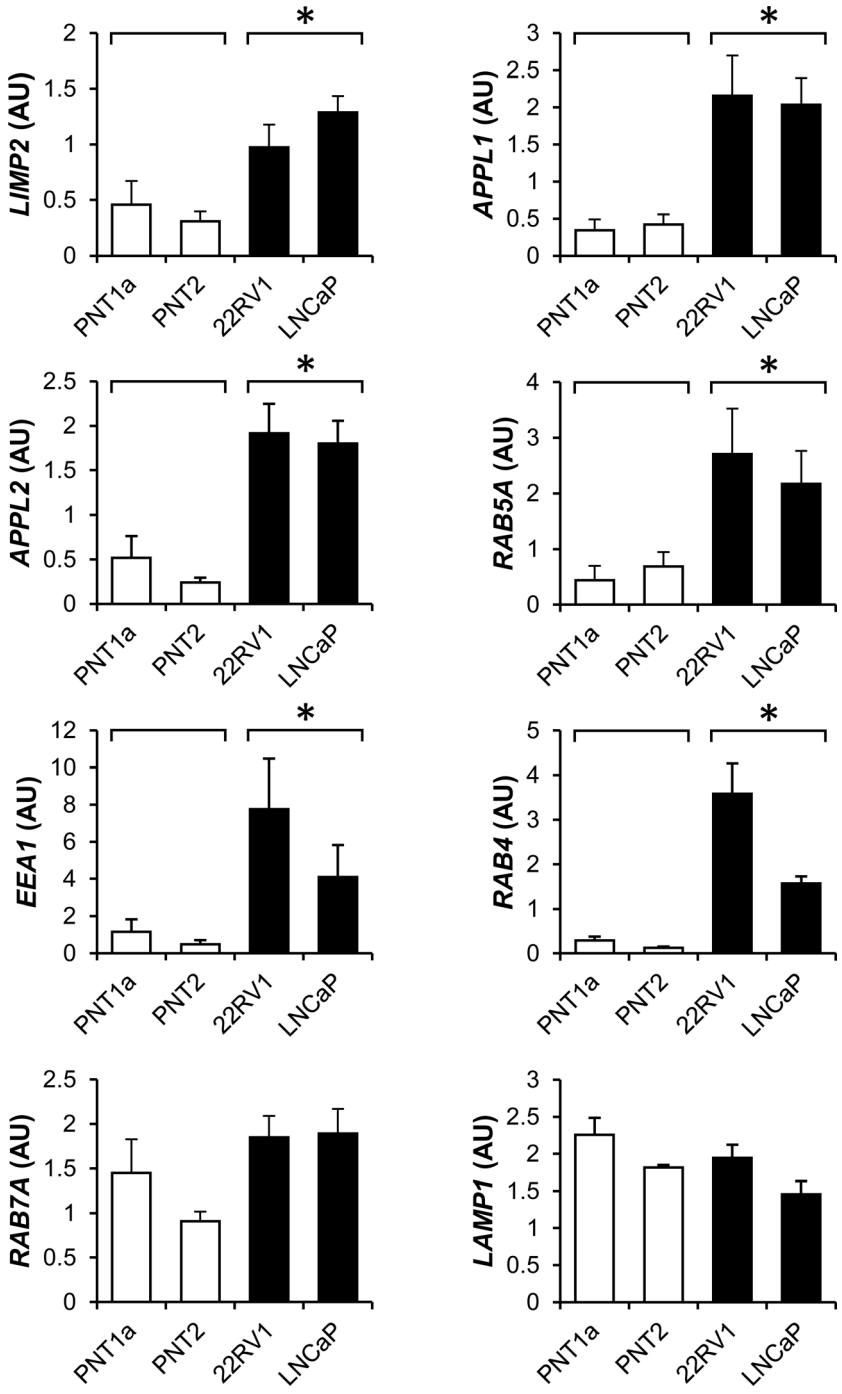


Figure 4.1 – Quantification of endosome and lysosome gene expression in non-malignant control and prostate cancer cell lines.

Levels of mRNA transcripts in prostate non-malignant control cell lines PNT1a and PNT2 (white bars), and cancer cell lines 22RV1 and LNCaP (black bars) were evaluated by qPCR in triplicate experiments. Data was expressed relative to *GAPDH* endogenous control and analysed by Kruskal-Wallis rank sum method. Statistical significance ($P \leq 0.05$) is represented by an asterisk.

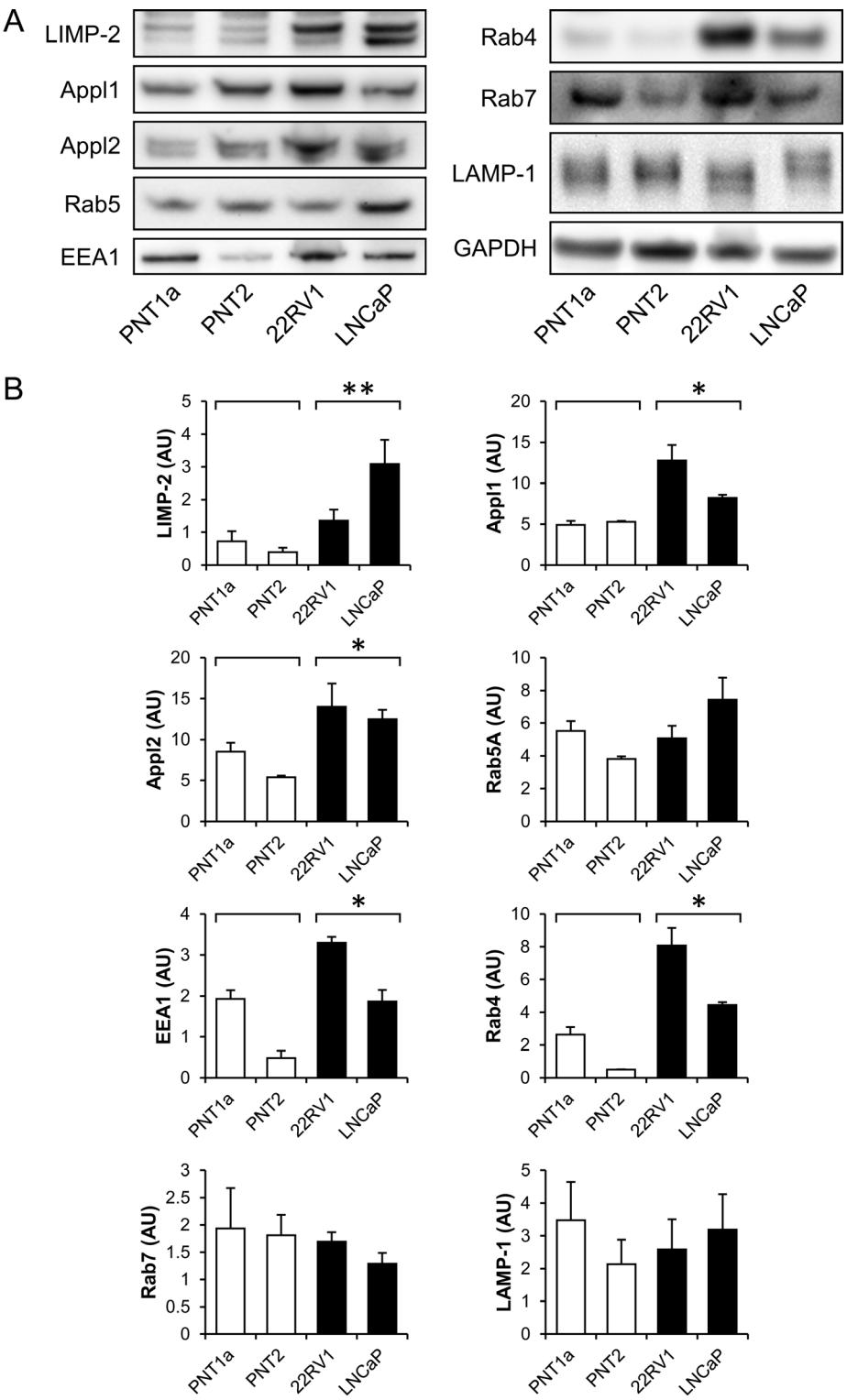

A
LIMP-2
Appl1
Appl2
Rab5
EEA1
Rab4
Rab7
LAMP-1
GAPDH
PNT1a
PNT2
22RV1
LNCaP
B
LIMP-2 (AU)
Appl1 (AU)
Appl2 (AU)
Rab5A (AU)
EEA1 (AU)
Rab4 (AU)
Rab7 (AU)
LAMP-1 (AU)
**
*

Figure 4.2 – Detection and quantification of intracellular lysosomal proteins in non-malignant control and prostate cancer cell lines.

(A) Representative images from Western blot analysis of 10 μg whole cell lysate from non-malignant control cell lines PNT1a and PNT2, and cancer cell lines 22RV1 and LNCaP, examined in triplicate. **(B)** Protein amount was quantified by densitometry relative to GAPDH protein endogenous control (arbitrary units). Data was analysed by Kruskal-Wallis rank sum method with statistical significance (** $P \leq 0.01$; * $P \leq 0.05$) represented by an asterisk.

4.2.2 Altered distribution of endosomes and lysosomes in prostate cancer cells.

Confocal microscopy, analysing the distribution of endosomes and lysosomes (Figure 4.3), showed increased staining and altered compartment distribution in prostate cancer compared to the non-malignant controls. In non-malignant control cells LIMP-2 was concentrated in the perinuclear region, with some tubular and punctuate vesicular staining in the remainder of the cytoplasm and near the cell surface. In contrast, prostate cancer cells displayed relatively smaller LIMP-2 compartments, which had an even distribution throughout the cytoplasm. Appl1 positive endosomes were detected mainly in the perinuclear region of non-malignant control cells, whereas in prostate cancer cells these compartments were distributed towards the cell periphery and tended to be more concentrated near the plasma membrane in cellular extensions/pseudopodia. Rab5A displayed a similar distribution to its effector EEA1 in both non-malignant control and prostate cancer cells. However, in non-malignant control cells both Rab5A and EEA1 were concentrated in the perinuclear region, while in prostate cancer cells these endosomal compartments were found throughout the cytoplasm, with some compartments located towards the cell periphery in cellular extensions. Rab7 positive endosomes were located mainly in the perinuclear region of both non-malignant control and prostate cancer cells. In non-malignant control cells, LAMP-1 was detected on compartments that were concentrated in the perinuclear region, whereas in prostate cancer cells the LAMP-1 compartments were distributed away from the perinuclear region, concentrated in cellular extensions. Consistent with the LAMP-1 staining, LysoTracker™ positive acidic compartments were concentrated mainly in the perinuclear region of non-malignant control cells, whereas in prostate cancer cells, these compartments were detected in both the perinuclear region and in cytoplasmic extensions (Figure 4.4).

LAMP-1
Rab7
Rab4
EEA1
Rab5A
Appl1
LIMP-2
PNT1a
PNT2
22RV1
LNCaP

Figure 4.3 – Confocal micrographs of endosome markers in prostate cell lines.

Fixed, non-malignant (PNT1a and PNT2) and prostate cancer cells (22RV1 and LNCaP), were probed for endosome markers (green), counterstained with DAPI nuclear stain (blue), and visualised by laser-scanning confocal microscopy. Micrographs from the co-staining of Rab5A and EEA1 are shown in the same cells for these respective markers. Transmitted light illumination was captured for visualisation of cell outline (white). Scale bars for all images represent 10 μm.

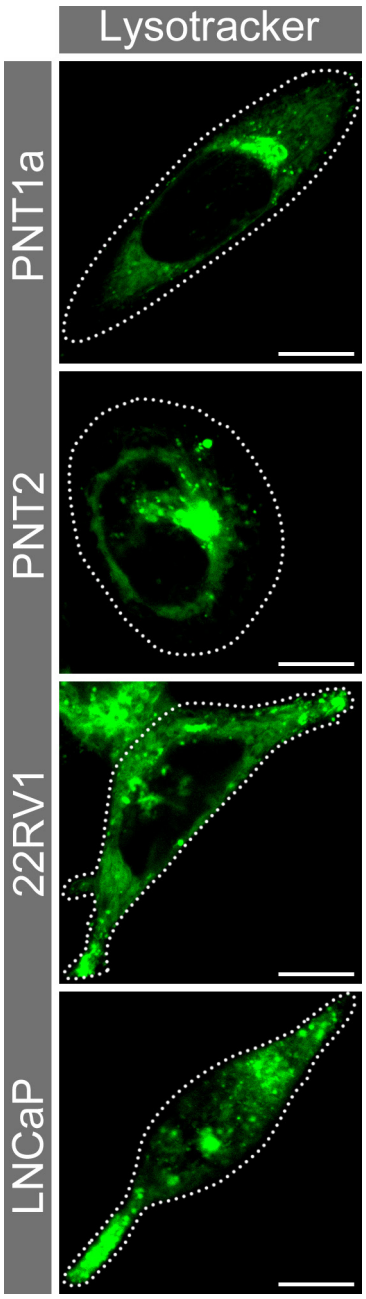


Figure 4.4 – Confocal micrographs of LysoTracker®-positive vesicles in prostate cell lines.

Non-malignant control prostate cell lines PNT1a and PNT2 and prostate cancer cell lines 22RV1 and LNCaP were stained with LysoTracker® (green). Cell outlines were visualised by transmitted light and membrane depicted by a white dotted line. Scale bars for all images represent 10 μm.

4.2.3 Altered distribution of endocytosed transferrin in prostate cancer cells.

Previous studies have reported increased uptake of transferrin in prostate cancer cells, prompting the investigation of receptor expression and transferrin endocytosis in relation to the observed increase in endosome protein expression and altered endosome distribution. In non-malignant control cells, endocytosed transferrin was observed in punctuate intracellular structures after 5 minutes and in the perinuclear region at 15 and 30 minutes (Figure 4.5). The prostate cancer cells endocytosed more transferrin than the non-malignant control cell lines and the internalised transferrin was more concentrated at the cell periphery of prostate cancer cells compared to a concentration in the perinuclear region of non-malignant controls (Figure 4.5). There was also a dramatic reduction in actin staining for the prostate cancer cell lines compared to the non-malignant control cell lines (Figure 4.5). In the non-malignant control cells, transferrin was clustered in LIMP-2 and Rab7 positive endosomes localised in the perinuclear region (Figure 4.6). While the prostate cancer cells had transferrin in some LIMP-2 positive endosomes in the perinuclear region and some co-localisation with the Golgi marker TGN46, the majority of transferrin was localised in different endosomal compartments that were Appl1, Rab5A and EEA1-positive, distributed throughout the cytoplasm and in cellular extensions (Figure 4.6). The Rab4 recycling endosomes and LAMP-1 positive lysosomes had similar patterns of transferrin staining for the prostate cancer and non-malignant control cell lines (Figure 4.6). Further analysis of the transferrin receptors revealed variable gene and protein expression for TfR1 and TfR2 (Figure 4.7). There was a significant increase in *TFR1* gene expression in prostate cancer cells when compared to non-malignant controls, but only a qualitative increase in TfR1 protein in the prostate cancer cell line 22RV1 and not for LNCaP (Figure 4.7 A). While there was significantly more TfR2 protein detected

in prostate cancer cells when compared to the non-malignant controls, there was only an increase in *TFR2* gene expression in the LNCaP cancer cells (Figure 4.7 A). Thus, while there was an increased amount of TfR protein in prostate cancer compared to non-malignant control cells, there was relatively more *TFR1* in 22RV1 and more *TFR2* in LNCaP cells.

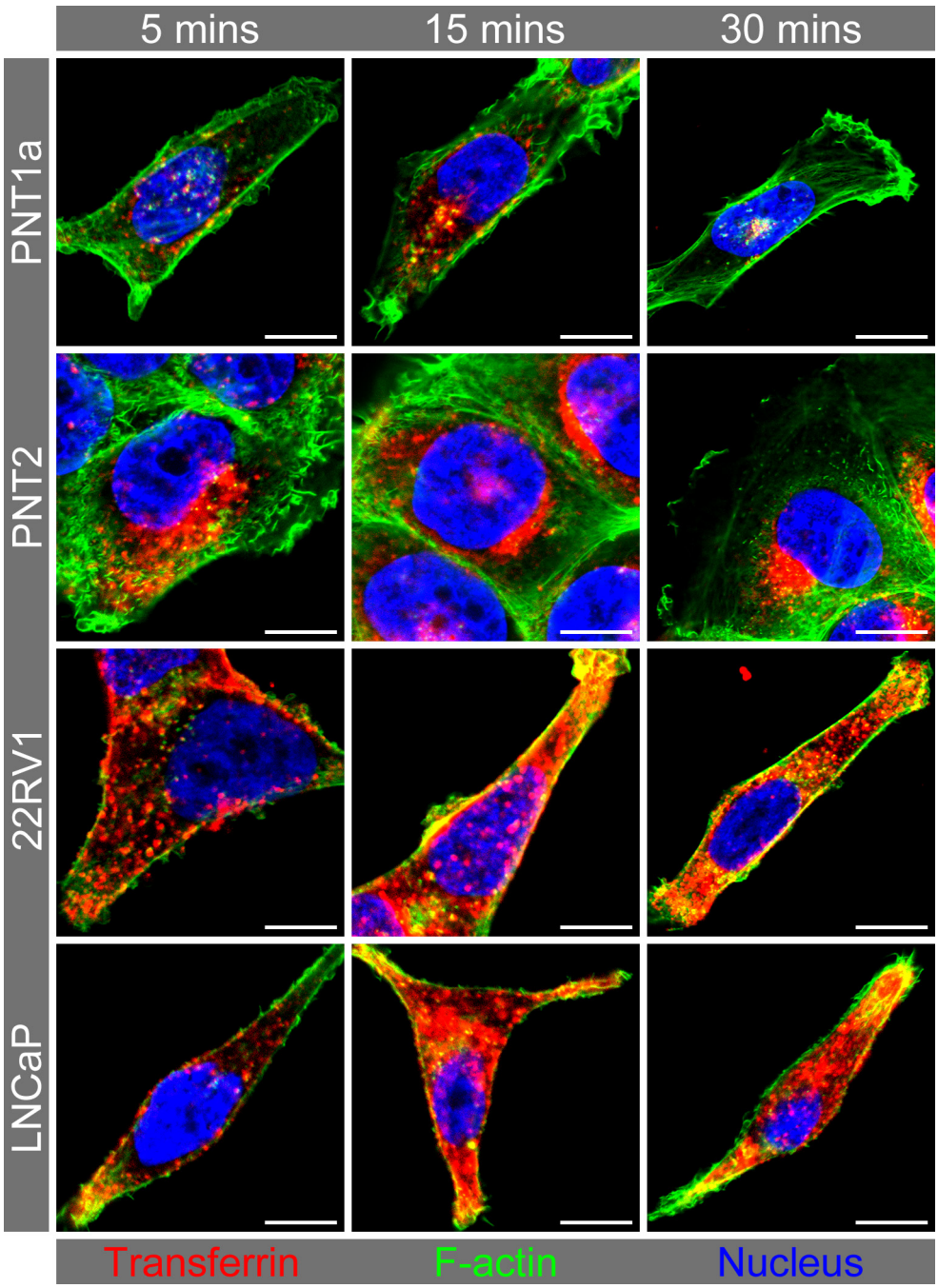


Figure 4.5 – Time-course of transferrin uptake in prostate cell lines.

Confocal micrographs showing increased uptake and altered distribution of transferrin in prostate cancer cell lines 22RV1 and LNCaP compared to non-malignant control cell lines PNT1a and PNT2. Cell cultures were incubated with transferrin Alexa Fluor® 633 conjugate (red) for a period of 5, 15 and 30 minutes prior to cell fixation and membranes labelled with phalloidin Alexa Fluor® 488 (green). Scale bars represent 10 μm.

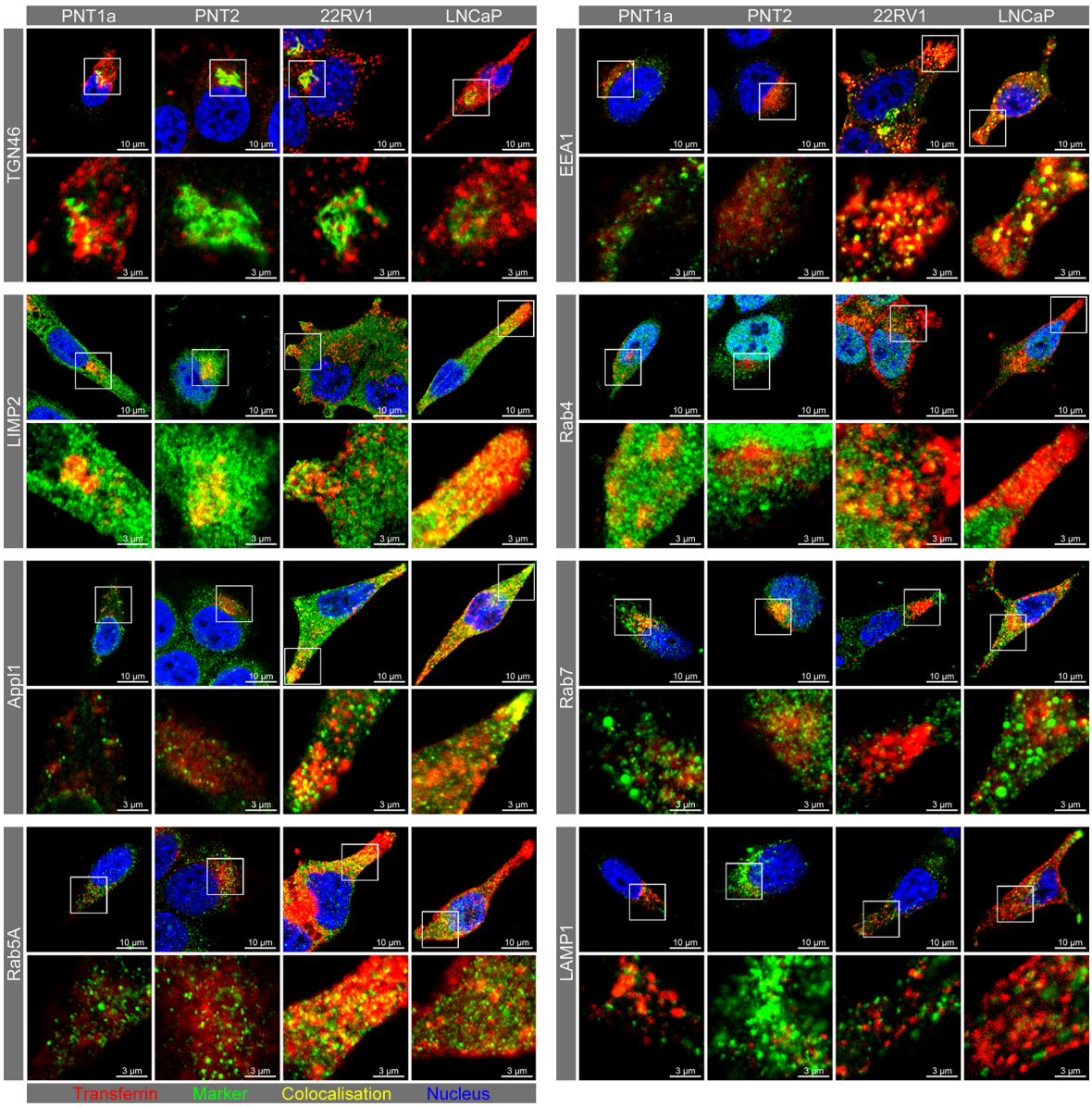


Figure 4.6 – Transferrin and endosome/lysosome marker colocalisation.

Confocal micrographs and enlargements showing transferrin (red) and an endosome/lysosome marker (green) in non-malignant control cell lines PNT1a and PNT2, and prostate cancer cell lines 22RV1 and LNCaP. Colocalisation is represented by yellow fluorescence.

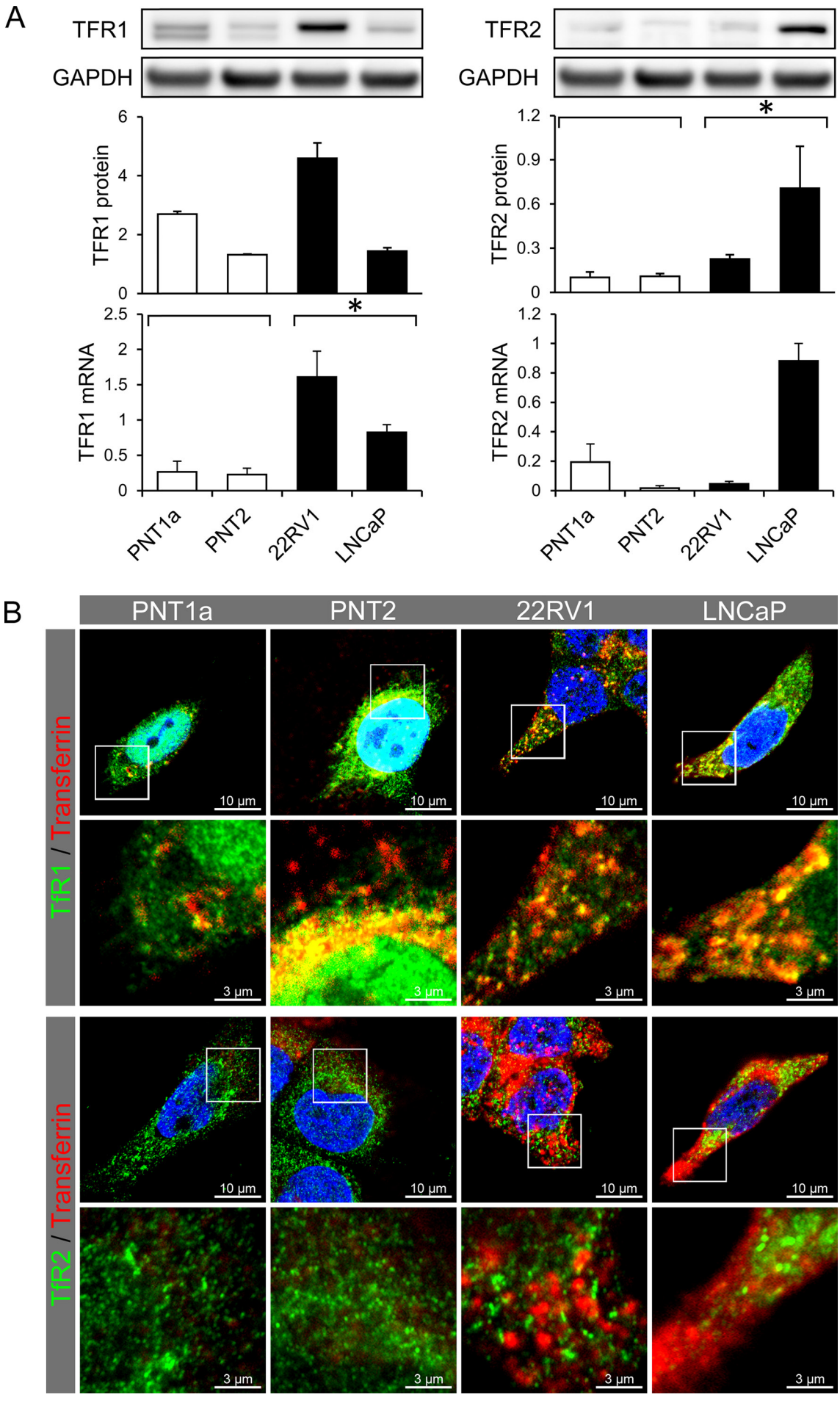

A
TFR1
GAPDH
TFR2
GAPDH
TFR1 protein
TFR2 protein
TFR1 mRNA
TFR2 mRNA
*
*
PNT1a
PNT2
22RV1
LNCaP
B
PNT1a
PNT2
22RV1
LNCaP
TfR1 / Transferrin
TfR2 / Transferrin
10 µm
3 µm

Figure 4.7 – Analysis of transferrin receptor expression and colocalisation with transferrin.

(A) Western blot analysis and quantification of protein and gene expression of transferrin receptor 1 (TFR1) and transferrin receptor 2 (TFR2). Quantification was performed from averages of triplicate experiments, relative to GAPDH protein and gene. Asterisk represents statistical significance ($P \leq 0.05$).

(B) Confocal micrographs and enlargements showing transferrin (red) and transferrin receptor (green) in non-malignant control cell lines PNT1a and PNT2, and prostate cancer cell lines 22RV1 and LNCaP. Colocalisation of transferrin receptor and transferrin is represented by yellow fluorescence.

4.2.4 Altered Akt signalling in prostate cancer cells.

The total amount of Akt protein detected in non-malignant control cells was similar to that detected in prostate cancer cells (Figure 4.8). There were, however, differences in the amount of phosphorylated Akt in the prostate cancer lines, with 22RV1 showing a marked reduction in the amount of phosphorylated Akt whereas LNCaP had an increased amount of phosphorylated Akt (Figure 4.8). More importantly, following the addition of transferrin, there was a significant five-fold increase in the amount of phosphorylated Akt in the PNT1a non-malignant control cell line, and an approximate two-fold increase of phosphorylated Akt in the PNT2 non-malignant control cell line, but no change in the amount of phosphorylated Akt in either of the cancer cell lines (Figure 4.8 B). Interestingly, the increased amount of phosphorylated Akt observed in the LNCaP cells (that had not been treated with transferrin), correlated with the amount of TfR2 detected in this cell line (Figure 4.7 A and Figure 4.8).

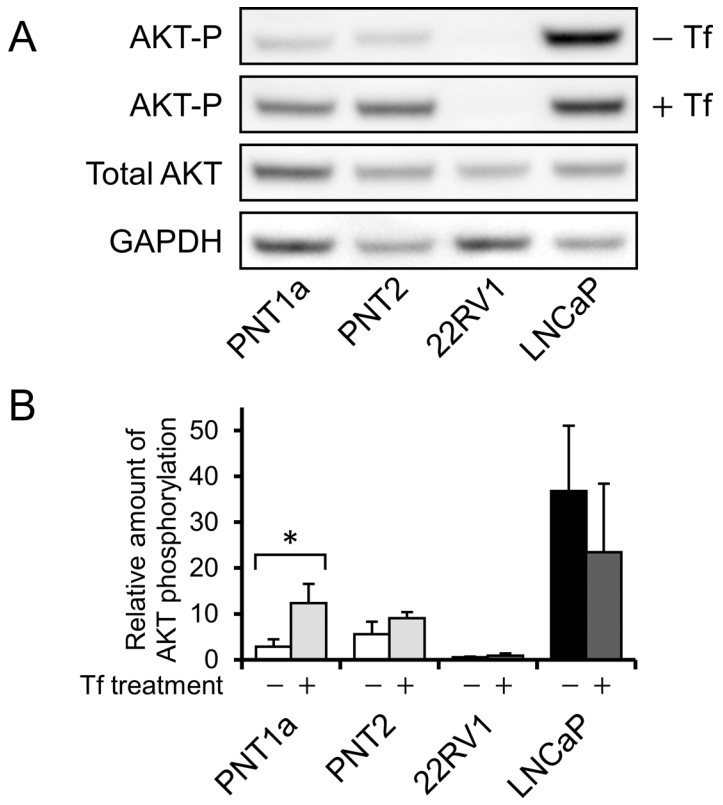


Figure 4.8 – AKT phosphorylation levels in non-malignant control and cancer cell lines before and subsequent to transferrin treatment

(A) 10 µg whole cell lysate from control cell lines PNT1a and PNT2, and cancer cell lines 22RV1 and LNCaP, was electrophoresed by SDS-PAGE and detected by Western blotting with anti-AKT and anti-AKT-P antibodies. **(B)** The change in the amount of phosphorylated AKT after treatment with transferrin was quantified relative to total AKT and GAPDH loading control. Statistical significance is marked by an asterisk (* $P \leq 0.05$).

4.2.5 Distribution of LAMP-1 and Appl1 in non-malignant and malignant human prostate tissue.

Immunohistochemistry was used to investigate the distribution of LAMP-1 and Appl1 in prostate cancer patient tissue samples. The lysosomal marker LAMP-1 showed some tumour specific staining in some patient samples (Figure 4.9), but consistent with previous studies, there were variable results with some cancer samples having little or no LAMP-1 staining (data not shown). Appl1, however, specifically delineated the cancer margins and showed dramatically increased staining within the tumour mass (Figure 4.9). This Appl1 staining pattern was consistent for each of the six different patient samples examined.

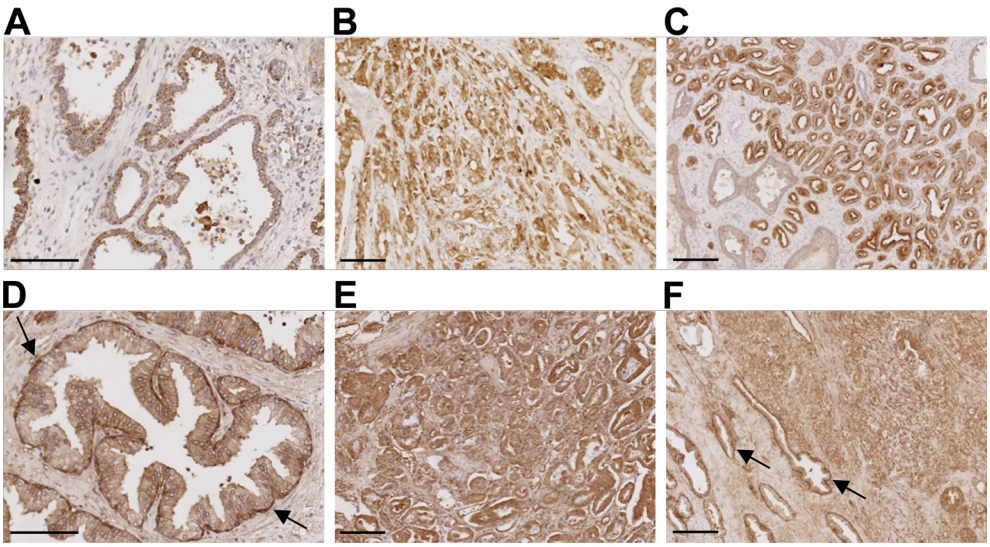


Figure 4.9 – LAMP-1 and Appl1 expression in prostate tissue isolated from different cases.

LAMP-1 and Appl1 expression was determined by immunohistochemistry in matched human non-malignant (A, D) and tumour (B, E) prostate tissue. Both non-malignant and malignant tissue is stained for LAMP-1 (C) and Appl1 (F). The arrows in D & F show Appl1 staining the basement membrane in non-malignant prostate tissue. Scale bar = 100 μM in A, B & D and 200 μM in C, E, F. Images acquired by Dr. Emma Parkinson-Lawrence.

4.3 Discussion

Prostate cancer is one of the most frequently diagnosed cancers in men and a leading cause of cancer related deaths world-wide, particularly in the United States and Australasian populations (AIHW 2012; Greenlee et al. 2000; Parkin et al. 2005). The prostate specific antigen is still a commonly used test to detect prostate cancer, but has significant problems in terms of miss-diagnosis and prognostic prediction (see for example: Roobol, Haese & Bjartell 2011; Stenman et al. 2005). Some promising adjunct tests have recently been developed including prostate cancer antigen 3 (PCA3) (Ploussard & de la Taille 2010), the analysis of cholesterol sulphate (Eberlin et al. 2010) and a novel sequence of the gene protein kinase C-zeta (*PRKCZ*) which is translated to the protein PRKC-ζ-$_{PrC}$ (Yao et al. 2012). However, these biomarkers do not provide an optimal method for the early and accurate detection of prostate cancer, which is needed to enable appropriate therapeutic intervention.

There have been extensive protein and proteomic studies to delineate potential new prostate cancer biomarkers (see for example: Goo & Goodlett 2010; Liu, AY, Roudier & True 2004; Roobol, Haese & Bjartell 2011; You et al. 2010), but despite this, the ideal marker with appropriate sensitivity and specificity is yet to be established. Interestingly, many of the early biomarkers investigated, and some of the recent proteins identified in proteomic studies, are either lysosomal hydrolases (e.g. lysosomal cathepsins, acid ceramidase and acid phosphatase), lysosomal membrane proteins (e.g. LAMP 1-3 also called CD107a, b and CD63) or proteins that are delivered from the cell surface into the endosome-lysosome system (e.g. sialomucin/CD164, CD1, CD47, CD75). Despite the strong association between lysosomal proteins, hydrolase release and extracellular matrix

degradation, there has not been a systematic analysis of endosome-lysosome biogenesis and how this critical organelle system might be functionally altered in prostate cancer cells.

Altered distribution of lysosomes has previously been reported in prostate cancer cells (Rempel et al. 1994; Sloane, B. F. et al. 1994). Moreover, acidic extracellular pH has been shown to exacerbate this phenomenon, stimulating the traffic of lysosomal organelles to the periphery of cancer cells (Glunde et al. 2003; Steffan, Joshua J. et al. 2009). This altered lysosome traffic has been linked with the release of cathepsin B and tumour invasiveness (Steffan, J. J. & Cardelli 2010), presumably due to hydrolysis of the extracellular matrix (Roshy, Sloane & Moin 2003; Steffan, Joshua J. et al. 2010). However, cathepsin B has been reported to be more enriched in endosomes (Authier, Kouach & Briand 2005; Authier et al. 1999) rather than lysosomes, whereas the reverse is true for another prostate cancer biomarker cathepsin D (Zaidi et al. 2008). The movement of lysosomal related vesicles to the periphery of prostate cancer cells has been shown to be dependent on GTPases (e.g. RhoA), microtubules via the molecular motor protein KIF5b, and to involve PI3K, Akt/Erk1/2 phosphorylation and MAPK signalling (Steffan, J. J. & Cardelli 2010). Moreover, a component of the MAPK signalling pathway, the endosome-localised MAPK/Erk Kinase (MEK1) p14-MP1 scaffolding complex, has been shown to specifically interact with and regulate the distribution of endosomes via ERK signalling (Deacon et al. 2005; Teis, Wunderlich & Huber 2002). Here we observed altered distribution of endosome and lysosome vesicles into the cellular periphery of prostate cancer cells. Increases in Na^+/H^+ exchange activity (acidification), RhoA GTPase activity and PI3K activation have been shown to result in exocytosis from prostate cancer cells (Steffan, Joshua J. et al. 2009). The increased endosomal associated gene and protein expression together with the previously observed cathepsin B release, suggested that endosome

related proteins may provide an important new focus for prostate cancer disease marker studies.

Increased expression of LIMP-2 has also been shown in oral squamous cell carcinoma and was associated with tumour metastasis (Pasini et al. 2012). LIMP-2 has been reported to have a role in endosome biogenesis and its overexpression evoked the enlargement of both early and late endosomes (Kuronita et al. 2002). We observed increased gene and protein expression of the endosomal protein LIMP-2 in prostate cancer cell lines, prompting us to further investigate endosomal biogenesis in prostate cancer cells. The early endosome associated proteins EEA1, Appl1, Appl2 and recycling endosome protein Rab4 were significantly upregulated in prostate cancer cells, supporting the hypothesis of altered endosome biogenesis in prostate cancer. Furthermore, the EEA1, Appl1 and Appl2 endosome sub-populations each displayed altered intracellular distribution consistent with altered endosome traffic and potentially function.

The significant changes that we observed in endosome associated gene and protein expression, together with the altered distribution of endosome populations led us to investigate transferrin receptor expression together with transferrin endocytosis, sorting and Akt signalling as measures of endosome function. Significant increases in the amount of transferrin receptor have previously been reported in prostate cancer cells (Keer et al. 1990), and this has been linked to c-Myc activation, which alters proliferation and tumourigenesis (O'Donnell et al. 2006). Akt signalling is also essential for regulating cell growth and survival; and this controls the cell surface expression of transferrin and growth factor receptors (Edinger & Thompson 2002). The transferrin receptor has previously been observed to colocalise with Rab5 and the motor protein myosin VI; the latter of which is

involved in retrograde transport to the plasma membrane (Puri et al. 2010). This was consistent with our observations of endosome populations co-staining with labelled transferrin in the cellular periphery of prostate cancer cells. There also appeared to be a deregulation of Akt signalling in the prostate cancer cells, which may have altered the intracellular location of the transferrin receptor, routing it into the different populations of Appl1, Appl2 and Rab5 endosomes.

Appl1 has been shown to be directly involved in insulin signalling and the translocation of the glucose transporter GLUT-4, which is mediated by direct binding of Appl1 to PI3K and Akt (Cheng et al. 2012; Wang, C et al. 2009), inducing endosome relocalisation. In prostate cancer cells, Appl1 potentiated Akt activity has also been shown to suppress androgen receptor transactivation (Yang, L et al. 2003). The increased gene and protein expression of Appl1 that we observed in prostate cancer cells might be expected to cause increased glucose uptake, due to its effect on GLUT-4 and this could have implications for energy metabolism in these cancer cells. Indeed, Appl1 also regulates other aspects of both lipid and glucose metabolism, activating AMP-activated kinase, p38 MAP kinase (MAPK) and PPARα (Deepa & Dong 2009; Wang, C et al. 2009). Appl2, which is homologous to Appl1, has been shown to function as a negative regulator of adiponectin signalling, by competitive binding with Appl1 for interaction with the adiponectin receptor, again regulating energy metabolism. The increased expression of both Appl1 and Appl2 could therefore heavily influence prostate cancer cell metabolism, particularly as these proteins were not observed together, but rather on separate populations of endosomes. This could have direct significance for increased energy utilisation and prostate cancer cell survival. The altered Appl1 expression and effect on Akt signalling in prostate cancer cells would be expected to also have significant consequence for other aspects of prostate cancer biology,

due to the importance of the Appl1/PI3K/Akt signalling pathway in leading cell adhesion and cell migration (Broussard et al. 2012). Notably, Appl1 also acts as a mediator of other signalling pathways, by interaction with the cytosolic face of integral or membrane associated proteins either at the cell surface or in the endosome pathway; where it is directly involved in endosome traffic.

Rab GTPases are integrally involved in the control of endosomal traffic, cycling between the cytoplasmic GDP bound state and the active membrane associated GTP bound state. Rab5 and Rab7 respectively define early and late endosome compartments and during endosome maturation, Rab5 recruits the HOPS complex as a mechanism to activate and be replaced by Rab7. mVps39 is known to be a guanine nucleotide exchange factor (GEF), which promotes the GTP bound state on endosomal Rabs, while TBC-2/TBC_1D_2 is a Rab GTPase activating protein (GAP) that promotes the GDP bound state; which in combination is used to regulate the membrane localisation of Rab proteins. TBC-2/TBC_1D_2 is thought to act as a regulator of endosome to lysosome traffic and is required to maintain the correct size and distribution of endosomes (Chotard et al. 2010). The altered distribution of endosome populations that we observed in prostate cancer cells suggests that TBC-2/TBC_1D_2 (GAP) and or mVps39 (GEF) might be functionally impaired. Interestingly, microarray analysis has detected increased expression of TBC-2/TBC_1D_2 and reduced Vps39 mRNA (Peralta, Martin & Edinger 2010). This altered GEF and GAP function has been shown to be critical for endosomal traffic of integrins and there have been direct links established between Rab GTPase activity and cancer progression (Pellinen et al. 2006; Subramani & Alahari 2010).

The expression of endosome markers has not previously been investigated thoroughly in prostate cancer, although some lysosomal related cell surface CD markers and LAMP-1/LAMP-2 have been utilised in tissue biopsy analysis. The Gleason grading system to define histological differentiation is used in conjunction with marker analysis to predict the course of disease in prostate cancer patients. The lysosomal membrane proteins like LAMP 1-3 and CD markers CD164, CD1, CD47 and CD75 are often evident in primary and metastatic cancer biopsies (Liu, AY, Roudier & True 2004), but their predictive capacity for disease progression is limited. We observed increased amounts of Appl1 protein in tissue biopsies from prostate cancer patients, confirming the increased gene and protein expression of Appl1 in prostate cancer cell lines. Further evaluation of this and the other markers that we have investigated is required to establish the validity and predictive value of endosome markers in prostate cancer.

We have demonstrated increased expression of early endosome markers and altered localisation of endosome and lysosome compartments in prostate cancer cells, which is associated with altered endocytosis and recycling of the transferrin receptor. We concluded that endosome biogenesis and function is altered in prostate cancer cells, opening a potentially new avenue of investigation, to develop markers that aid in the diagnosis and prognosis of prostate cancer.

Chapter 5

Microarray expression profiling of endosome and lysosome genes *in vivo*

5.1 Introduction

The observations of altered endosome biology in prostate cancer cells suggested that the increased expression of specific endosomal genes and proteins might critically influence the development and or progression of prostate cancer. Altered endosome biogenesis may therefore provide a new avenue for the investigation of biomarkers that can be used for prostate cancer diagnosis and prognosis. However, it was important to correlate the *in vitro* observations with patient data, to establish that the novel changes are biologically relevant. Verifying that there was altered endosome-related gene expression in prostate cancer microarray databases was viewed as an important first step in this process.

Gene microarray databases enable the investigation of biomarker expression in patients and determine any relation to known clinical parameters, which may then be used to predict clinical outcome (Aaltomaa et al. 2005; Markert et al. 2011; Thompson, VC et al. 2012). Microarrays have previously been used to produce expression profiles that compare prostate cancer to benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and normal prostate tissue (reviewed by Sorensen & Orntoft 2010). For example, the enzyme α-Methylacyl-CoA racemase (*AMACR*) is highly expressed in prostate cancer, and may potentially be used as a diagnostic or prognostic marker for the disease (Rubin et al. 2002). However, prostate cancers display heterogeneity and the altered expression of a single gene may not be indicative of the wider prostate cancer population. In addition, gene expression may alter throughout the course of the cancer growth and differentiation; for example, α-Methylacyl-CoA racemase is an androgen-regulated gene and exhibits variable expression upon androgen-deprivation therapies or androgen-independent disease progression (Vaarala et al. 2012). Therefore, multiple genes may be required to improve the accuracy of prostate cancer diagnosis and prognosis, effectively using a gene signature.

These gene signatures can be investigated in microarray databases to help retrospectively define accuracy/specificity of detection and to establish whether the biomarkers are informative for clinical outcome.

To analyse endosome-lysosome gene expression in relation to patient outcome a range of prostate cancer microarray databases were selected for investigation. Multiple microarray cohorts are available for analysis from Oncomine[10] (Life Technologies Pty., Ltd.) or the Gene Expression Omnibus[11]. A recent cohort of patients treated by radical prostatectomy at the Memorial Sloan-Kettering Cancer Center (MSKCC) was also selected (Taylor et al. 2010) and herein referred to as the Taylor cohort. This cohort comprised 150 primary prostate cancer and 29 matched non-malignant control samples. For the Taylor cohort, specimens were collected and snap-frozen prior to the identification of cancer regions; RNA extraction was performed on samples containing greater than 70 % cancer-cell content, based on histological assessment. Analysis was performed using an Affymetrix Human Exon 1.0 ST array[12] with the resulting microarray data obtained from the cBio Cancer Genomics Pathway Portal[13]. This cohort was also selected because it contained expression data for all of the relevant endosome and lysosome genes that were analysed here, *in vitro*. Furthermore, the Affymetrix GeneChip® used in this cohort was created from sequences derived from the RefSeq database build 34 (July 2003)[14], which provides greater sequence accuracy than previous microarrays using the Affymetrix GeneChip® HGU95 (Chandran et al. 2007; Mecham et al. 2004). An additional microarray by Tomlins et al. was also analysed and herein referred to as the Tomlins cohort, which was generated using

[10] oncomine.com (accessed October 2012)
[11] ncbi.nlm.nih.gov/geo (accessed October 2012)
[12] affymetrix.com/browse/products.jsp?productId=131452 (accessed June 2012)
[13] cbio.mskcc.org/cancergenomics-dataportal (accessed June 2012)
[14] genome.ucsc.edu/cgi-bin/hgGateway?hgsid=303107085 (accessed September 2012)

alternative microarray technology; specifically, a non-commercial custom-made array (Tomlins et al. 2007). This cohort comprised of non-malignant prostate tissue (n = 27), prostatic intraepithelial neoplasia (n = 13), primary cancer (n = 32) and metastatic cancer tissue (n = 20). Thus, in addition to confirming the results obtained for the Taylor cohort, the Tomlins cohort enabled the assessment of gene expression during disease progression from prostatic intraepithelial neoplasia through to metastatic cancer, which may be diagnostically relevant. Prostate samples for the Tomlins cohort were collected using laser-capture micro-dissection (Bonner et al. 1997) with approximately 10,000 cells collected from each sample. This method of sample collection was advantageous since specificity to specific sample type can be increased, with tissue surrounding the cancer excluded from capture, leading to cleaner RNA preparation and analysis (Bonner et al. 1997; Shekouh et al. 2003). The prostate cancer patient tissue analysed by Glinsky et al. included cancer patients that were monitored over the course of five years from initial cancer detection, to determine recurrence based on PSA concentration post-therapy (Glinsky et al. 2004); and therefore provided a capacity to determine the prognostic capability of endosome or lysosome genes, by analysing expression during cancer progression. The analysis of these different microarray databases aimed to verify the altered gene expression observed *in vitro* using cultured cells, as well as to provide some indication of the consistency for marker expression in patient samples.

5.2 Results

5.2.1 Altered expression of lysosome-related genes in prostate cancer was independent of TFEB expression.

The transcription of lysosome-related genes such as *LIMP2* that influence endosome and lysosome biogenesis is regulated by the transcription factor EB (TFEB) (Palmieri et al. 2011; Sardiello et al. 2009). Thus, *TFEB* gene expression may provide evidence of altered endosome and lysosome biology *in vivo.* Expression of *TFEB* was unchanged in prostate cancer compared to non-malignant control prostate tissue (Figure 5.1), whilst expression of *LIMP2* was significantly elevated in prostate cancer compared to non-malignant tissue ($P \leq 0.0001$). Conversely, *LAMP1*, which is also a target of *TFEB,* had significantly reduced expression in prostate cancer compared to non-malignant control tissue ($P \leq 0.01$). Other *TFEB*-regulated lysosomal genes were analysed to determine if their expression was altered independently of this transcription factor (Figure 5.2). Cathepsin B (*CTSB*) showed significantly reduced expression in prostate cancer tissue when compared to non-malignant control tissue using the Taylor cohort ($P \leq 0.0001$), whilst α-Glucosidase (*GAA*) had significantly elevated expression in prostate cancer compared to non-malignant control tissue ($P \leq 0.05$). The expression of acid ceramidase, β-glucosidase (*GBA*) and α-galactosidase A (*GLA*) were not significantly altered in prostate cancer tissue in the Taylor cohort when compared to non-malignant control tissue.

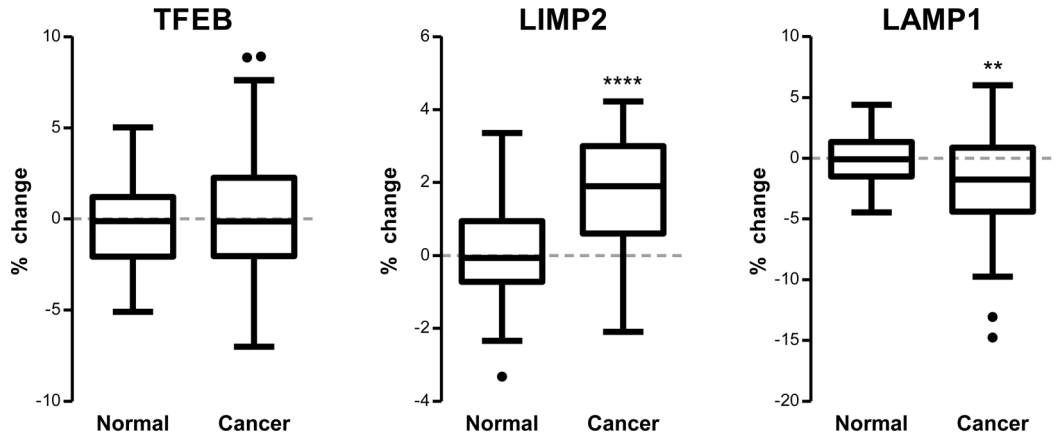


Figure 5.1 – Box-and-whisker graphs of *TFEB*, *LIMP2* and *LAMP1* gene expression. *LIMP2* and *LAMP1* gene expression is altered independently of *TFEB* expression in prostate cancer. Expression profiling data derived from Affymetrix Human Exon 1.0 ST arrays of 150 primary prostate cancers and 29 non-malignant tissues (Taylor et al. 2010) were quantitated to show percentage change of gene expression of *TFEB*, *LIMP2* and *LAMP1*. Box-and-whisker graphs were plotted with Tukey outliers (black points). Statistical significance is represented by an asterisk (** $P \leq 0.01$; **** $P \leq 0.0001$).

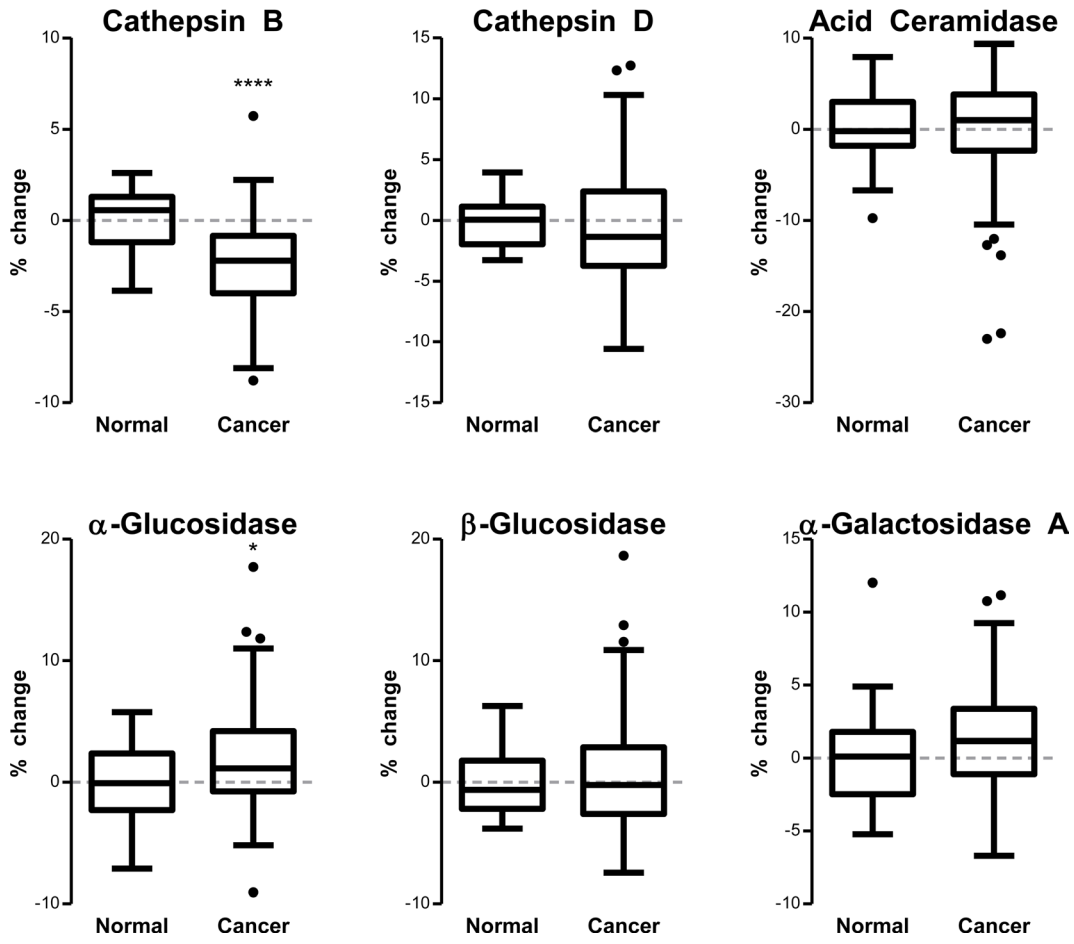


Figure 5.2 – Box-and-whisker graphs of *TFEB*-regulated lysosomal gene expression. *TFEB*-regulated lysosomal genes show variable expression in prostate cancer; cathepsin B had significantly reduced expression whilst α-glucosidase had increased expression in prostate cancer. Expression profiling data derived from Affymetrix Human Exon 1.0 ST arrays of 150 primary prostate cancers and 29 non-malignant tissues (Taylor et al. 2010) were quantitated to show percentage change of expression of lysosome-related genes. Box-and-whisker graphs were plotted with Tukey outliers (black points). Statistical significance is represented by an asterisk (* $P \leq 0.05$; **** $P \leq 0.0001$).

5.2.2 APPL1 had significantly elevated expression in primary prostate cancer tissue.

APPL1 gene expression was significantly elevated in prostate cancer tissue from the Taylor cohort when compared to non-malignant control tissue ($P \leq 0.05$; Figure 5.3). However, while elevated expression of *APPL2*, *RAB5A*, *EEA1*, *RAB4A* and *RAB7A* were observed *in vitro* (chapter 4.2.1), there was no significant difference in expression of these endosome related genes in primary prostate cancer compared to non-malignant prostate tissue (Figure 5.3).

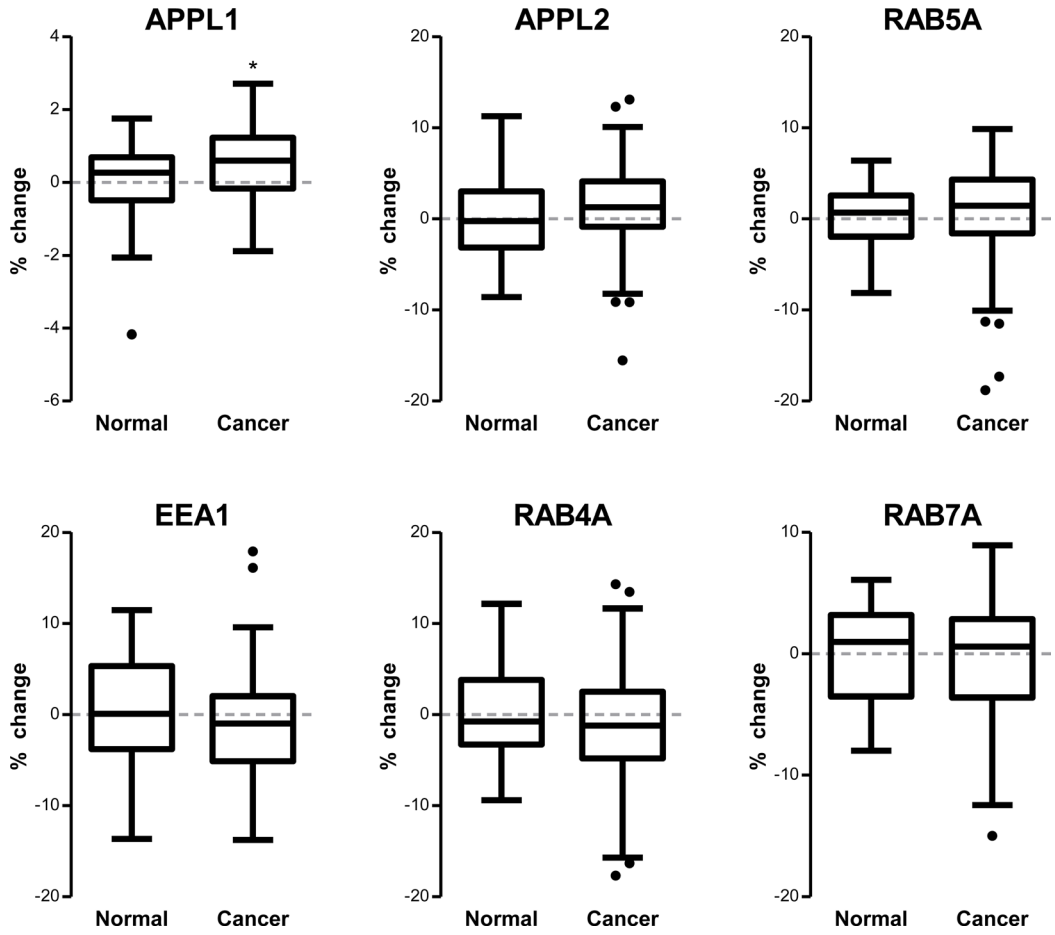


Figure 5.3 – Box-and-whisker graphs of endosomal gene expression.

APPL1 gene expression was significantly increased in prostate cancer whilst other endosome-related markers showed variable expression. Expression profiling data derived from Affymetrix Human Exon 1.0 ST arrays of 150 primary prostate cancers and 29 non-malignant tissues (Taylor et al. 2010) were quantitated to show percentage change of expression of endosome-related genes. Box-and-whisker graphs were plotted with Tukey outliers (black points). Statistical significance is represented by an asterisk (* $P \leq 0.05$).

5.2.3 Lysosomal and early endosome-related gene expression was altered during cancer progression.

The expression of endosome and lysosome related mRNA transcripts was also analysed from the Tomlins cohort enabling the analysis of prostatic intraepithelial neoplasia (PIN) and metastatic cancer tissue in addition to primary cancer and non-malignant tissue. The expression data for some genes such as *TFEB* was unavailable due to the custom microarray technology used and more limited probe-set compared to the commercial microarray used by Taylor et al. The gene expression of *LIMP2* and *LAMP1* in the Tomlins cohort showed no significant change between non-malignant control and primary prostate cancer tissue (Figure 5.4). Other *TFEB*-regulated genes displayed differential expression in the prostate cancer tissue when compared to non-malignant control tissue; cathepsin B was significantly reduced in primary cancer tissue compared to non-malignant tissue ($P \leq 0.05$), however there was no significant change in the expression of cathepsin D, acid ceramidase, α-glucosidase or α-galactosidase A between non-malignant tissue and primary cancer tissue from the Tomlins microarray (Figure 5.4). No expression data was available for β-glucosidase in this cohort.

Analysis of gene expression through the progressive disease states revealed a significant increase in *LIMP2* expression in prostatic intraepithelial neoplasia (PIN) ($P \leq 0.05$; Figure 5.4); variations of *LIMP2* expression were observed in metastatic tissue, however the mean reduction was not statistically significant. *LAMP1* expression was elevated in PIN and primary cancer compared to non-malignant tissue, with expression in metastatic tissue significantly reduced when compared with PIN ($P \leq 0.05$) and primary prostate cancer tissue ($P \leq 0.001$). Cathepsin B expression in primary prostate cancer and metastatic tissue was significantly downregulated when compared to non-malignant control tissue ($P \leq 0.05$

and $P \leq 0.001$ respectively). Cathepsin D expression showed some reduction through PIN, primary cancer and metastatic disease states, however this was not statistically significant when compared with non-malignant control tissue. In primary cancer tissue, acid ceramidase displayed similar expression to that observed in non-malignant control tissue; however, PIN tissue expression was significantly elevated when compared to non-malignant control and primary cancer tissue ($P \leq 0.01$ and $P \leq 0.05$ respectively). The expression of *ASAH1* was significantly reduced in metastatic prostate tissue when compared to non-malignant control ($P \leq 0.01$), PIN ($P \leq 0.001$) and primary cancer tissue ($P \leq 0.001$).

APPL1 expression was significantly increased in primary prostate cancer compared to non-malignant control tissue ($P \leq 0.05$; Figure 5.5), although there was no significant change in expression of *APPL1* between metastatic prostate tissue and non-malignant tissue. The expression of *APPL2* was significantly elevated in PIN and primary prostate cancer compared to non-malignant control tissue ($P \leq 0.05$ and $P \leq 0.01$ respectively); while metastatic tissue expressed similar amounts of *APPL2* to non-malignant tissue, and this was significantly reduced when compared to PIN and primary prostate cancer tissue ($P \leq 0.01$ and $P \leq 0.001$ respectively). The expression of *RAB5A* was significantly reduced in metastatic prostate tissue when compared to primary prostate cancer tissue ($P \leq 0.05$); however, there was no significant change in expression between non-malignant, PIN and primary prostate cancer tissue. The expression of *EEA1* was significantly elevated in primary cancer compared to non-malignant tissue ($P \leq 0.01$); whereas a significant reduction was observed in *EEA1* expression for metastatic tissue compared to primary prostate cancer ($P \leq 0.001$) and PIN tissue ($P \leq 0.05$). The expression of *RAB4A* was similar in primary prostate cancer compared to non-malignant control tissue; and the expression

of *RAB4A* in PIN tissue was significantly elevated when compared to primary prostate cancer tissue ($P \leq 0.05$). There was a significant reduction in the expression of *RAB4A* in metastatic prostate tissue when compared to non-malignant control ($P \leq 0.001$), PIN ($P \leq 0.001$) and primary prostate cancer tissue ($P \leq 0.01$). There was no significant change in the expression of *RAB7A* observed in PIN, primary cancer or metastatic cancer from the Tomlins microarray when compared to non-malignant control tissue.

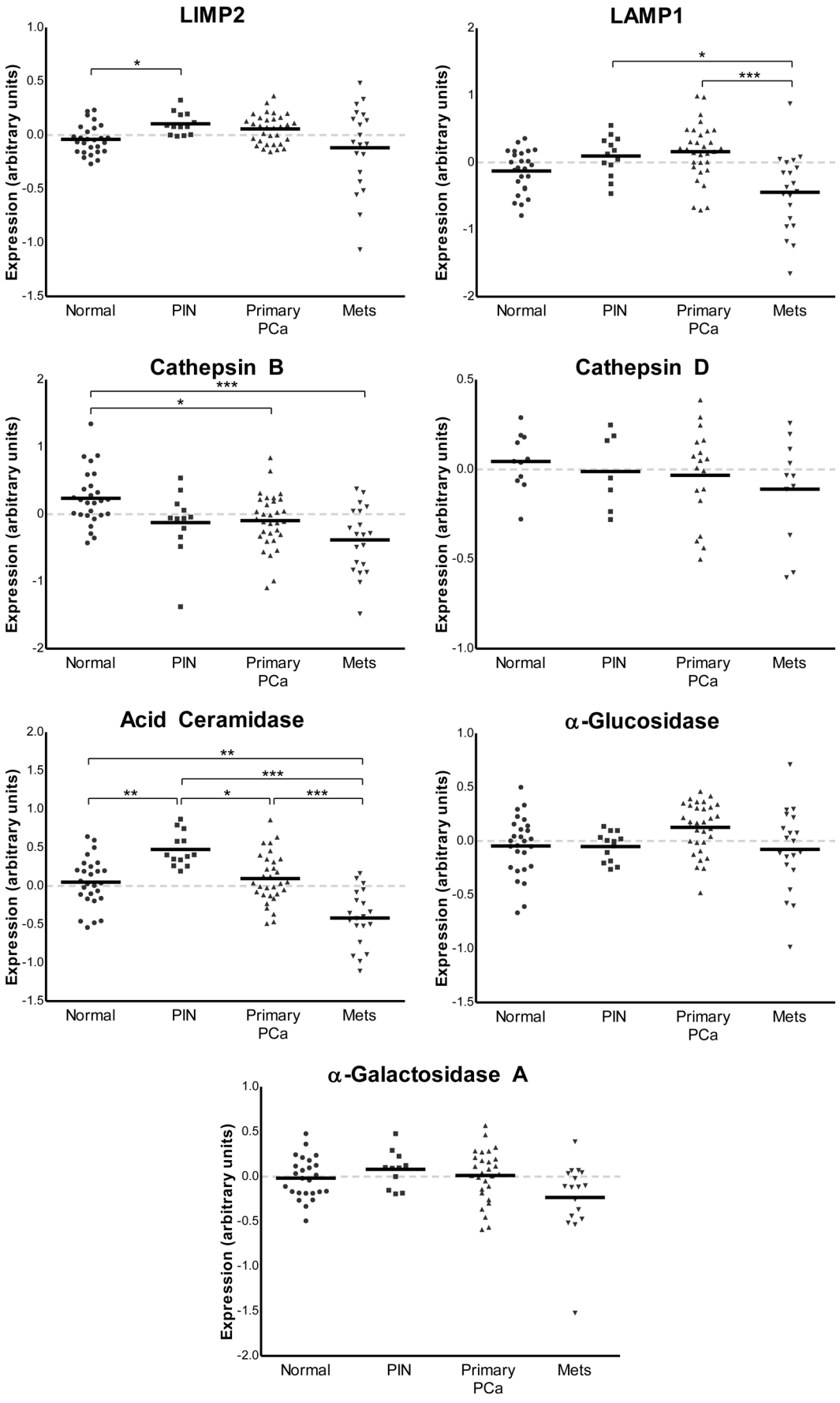

LIMP2
LAMP1
Cathepsin B
Cathepsin D
Acid Ceramidase
α-Glucosidase
α-Galactosidase A
Expression (arbitrary units)
Normal
PIN
Primary PCa
Mets

Figure 5.4 – Vertical scatter plots of lysosomal gene expression data from the cohort by Tomlins et al.

Detailed analysis of prostate cancer tissue revealed altered endosomal gene expression during prostate cancer progression. Expression profiling data derived from the Chinnaiyan Human 20K Hs6 array of 27 non-malignant tissues, 13 prostatic intraepithelial neoplasias, 32 primary prostate cancer and 22 metastatic cancer tissue samples (Tomlins et al. 2007) were quantitated to show relative amount of expression of lysosome-related genes. Statistical significance is represented by an asterisk (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

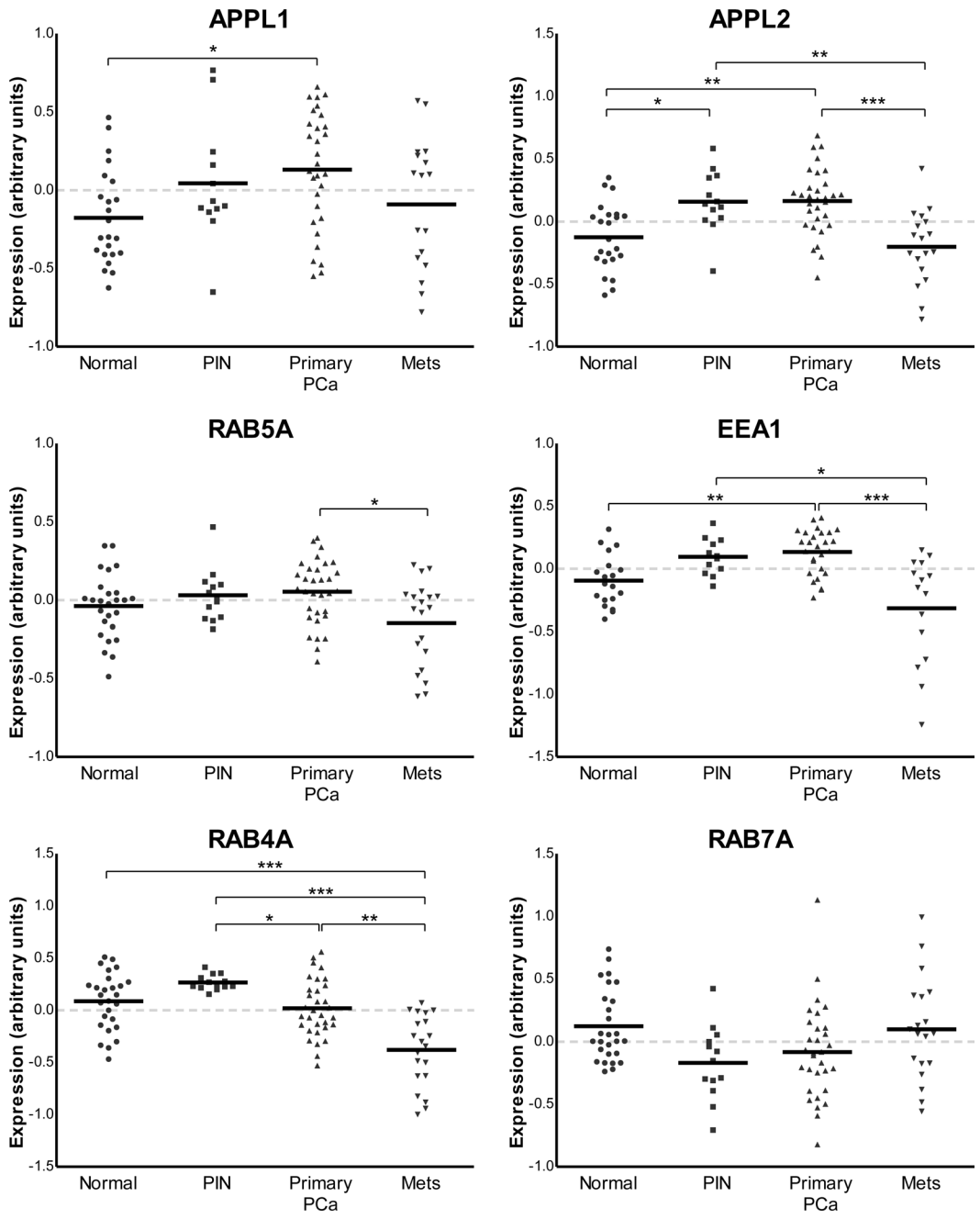


Figure 5.5 – Vertical scatter plots of endosomal gene expression data from the cohort by Tomlins et al.

Expression of endosomal genes in the Tomlins cohort showed significant increases, while metastatic prostate cancer tissue showed variable endosomal gene expression. Expression profiling data derived from the Chinnaiyan Human 20K Hs6 array of 27 non-malignant tissues, 13 prostatic intraepithelial neoplasias, 32 primary prostate cancer and 22 metastatic cancer tissue samples (Tomlins et al. 2007) were quantitated to show relative amount of endosome gene expression. Statistical significance is represented by an asterisk (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$)

5.2.4 Cathepsin B appeared to show some prognostic value for survival outcome in prostate cancer patients.

The quantification of gene expression may be a valuable tool for prostate cancer prognosis, as expression of some lysosome and endosome genes vary through PIN, primary cancer and metastatic cancer progression. To assess the prognostic potential of lysosomal genes, patients were classified from the Glinsky cohort (Glinsky et al. 2004) into two groups using *K*-means clustering based on gene expression. The Glinsky cohort microarray provides data of initial PSA blood concentration detected upon biopsy, and information on relapse subsequent to therapy. Clustering of high or low cathepsin B expression revealed patients with low amounts of cathepsin B expression who had significantly increased risk (P = 0.0374) of biochemical recurrence (Figure 5.6). The grouping by high or low expression for *LIMP2*, *LAMP1*, cathepsin D, acid ceramidase or α-galactosidase A genes did not stratify patients significantly into prognostic groups, however there was a trend observed for *LAMP1* and α-galactosidase A (P = 0.0623 and P = 0.11 respectively; Figure 5.6). From the increased expression of early endosome-related genes observed in the Taylor and Tomlins cohorts, we analysed the potential of these early endosome-related genes to stratify patients into prognostic groups (Figure 5.7). However, classifying patients into two groups based on expression of individual early endosome genes by *K*-means clustering did not significantly separate patients into prognostic groups.

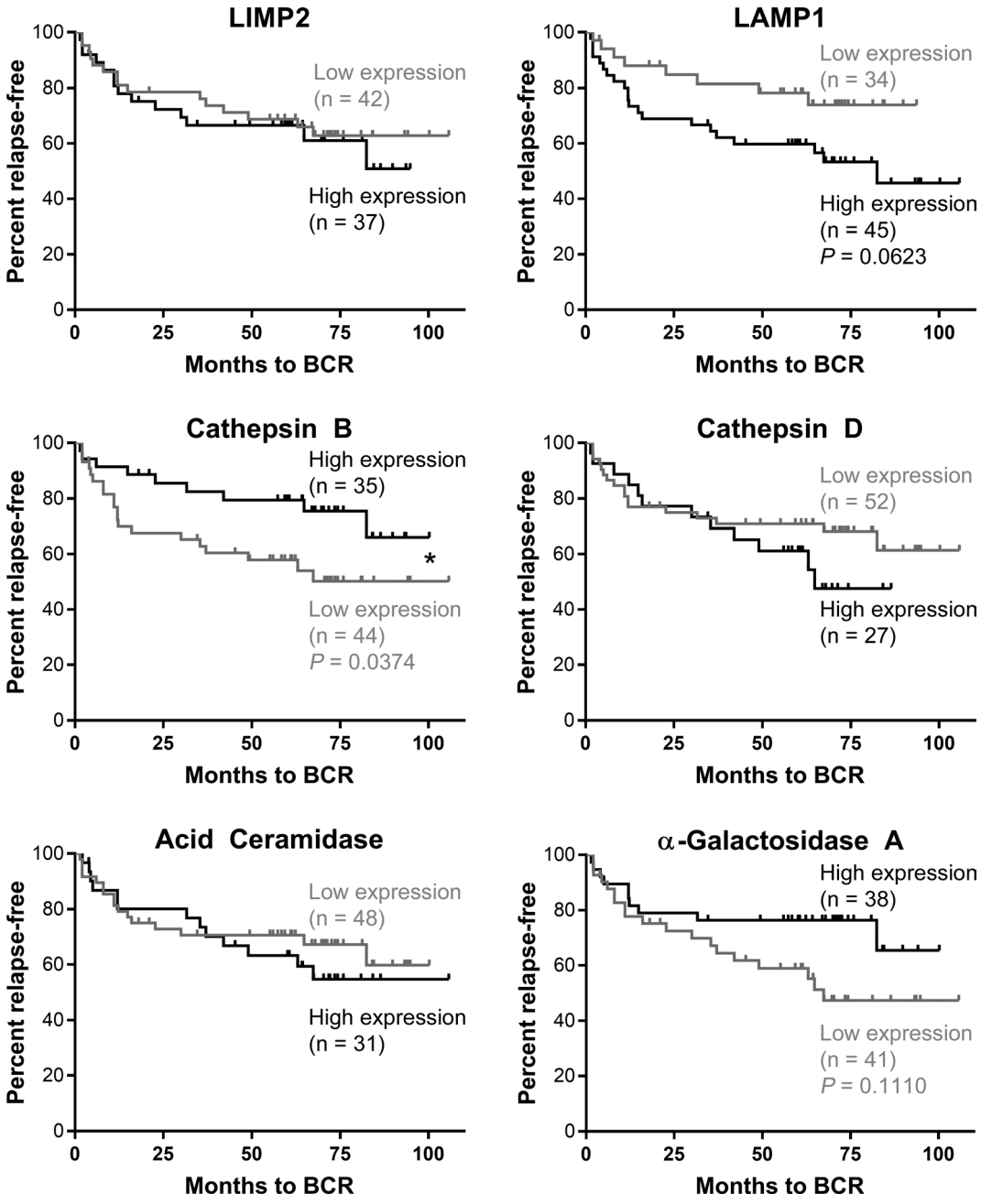


Figure 5.6 – Kaplan-Meier analysis of lysosomal genes and patient stratification based on biochemical recurrence (BCR).

Cathepsin B differentiated patients at risk of relapse based on the amount of gene expression. Expression of lysosomal genes that have previously been observed to be significantly altered in primary cancer showed trends for differences between high and low-risk patients, but were not significant. Patients from the Glinsky cohort (Glinsky et al. 2004) were stratified into two groups by *K*-means clustering based on gene expression level (high – black line, low – grey line). Statistical analysis was performed using Gehan-Breslow-Wilcoxon test.

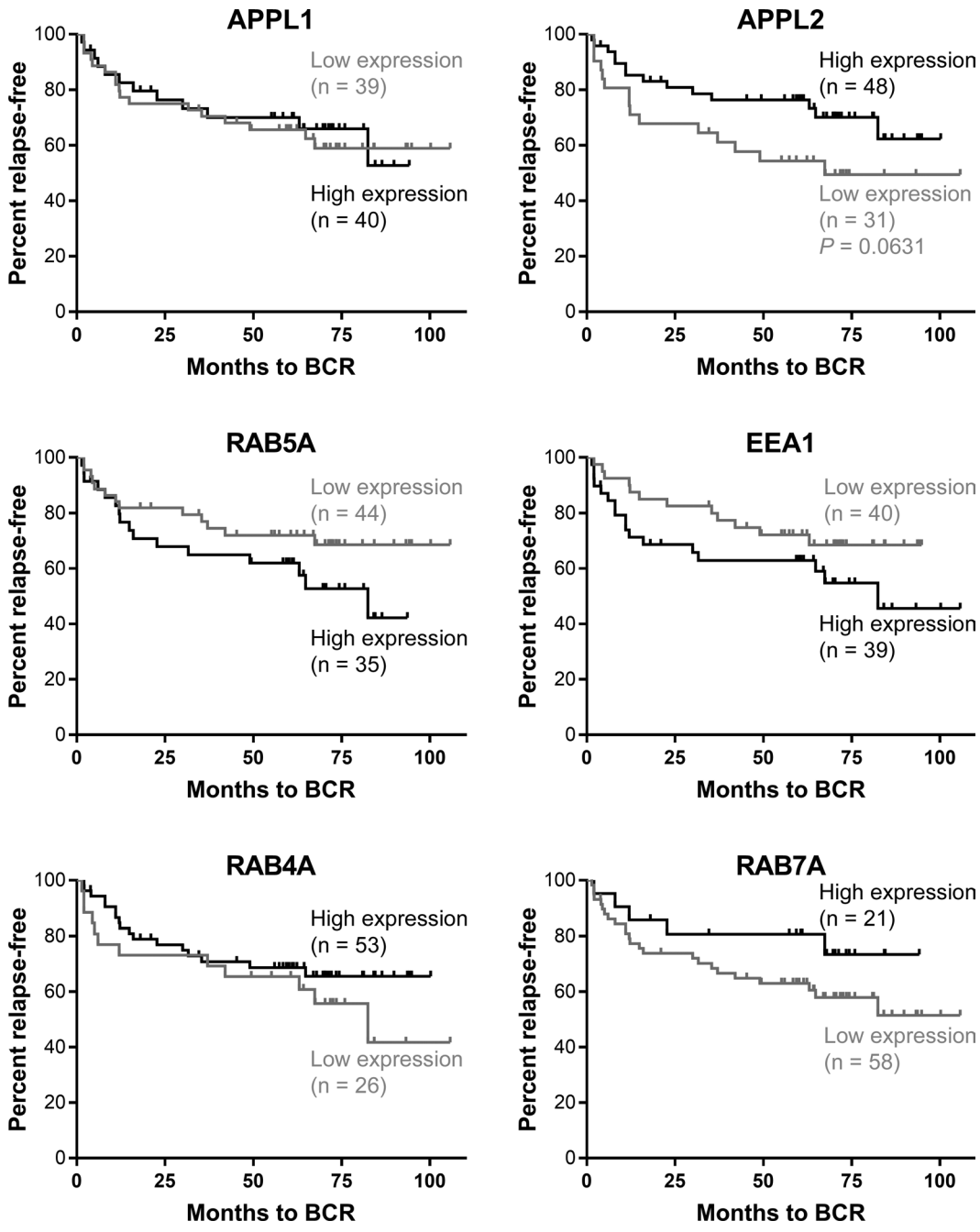


Figure 5.7 – Kaplan-Meier analysis of endosomal genes and patient stratification based on biochemical recurrence (BCR).

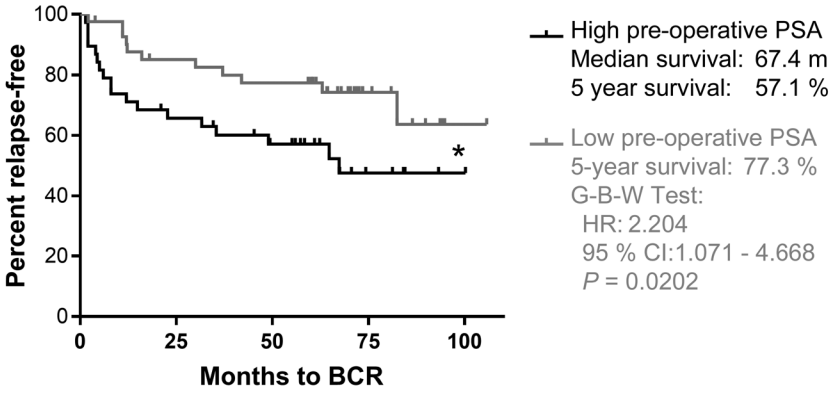
Endosomal gene expression did not stratify patients into significant prognostic groups. Patients from the Glinsky cohort (Glinsky et al. 2004) were stratified into two groups by *K*-means clustering based on amount (high – black line, low – grey line) of *APPL1*, *APPL2*, *RAB5A*, *EEA1*, *RAB4A* and *RAB7A* gene expression. Analysis was performed using Gehan-Breslow-Wilcoxon test; and no statistical significance was observed between the expression groups.

5.2.5 Kaplan-Meier analysis of lysosome and endosome gene expression shows significant prognostic value in cancer patients expressing low amounts of PSA protein.

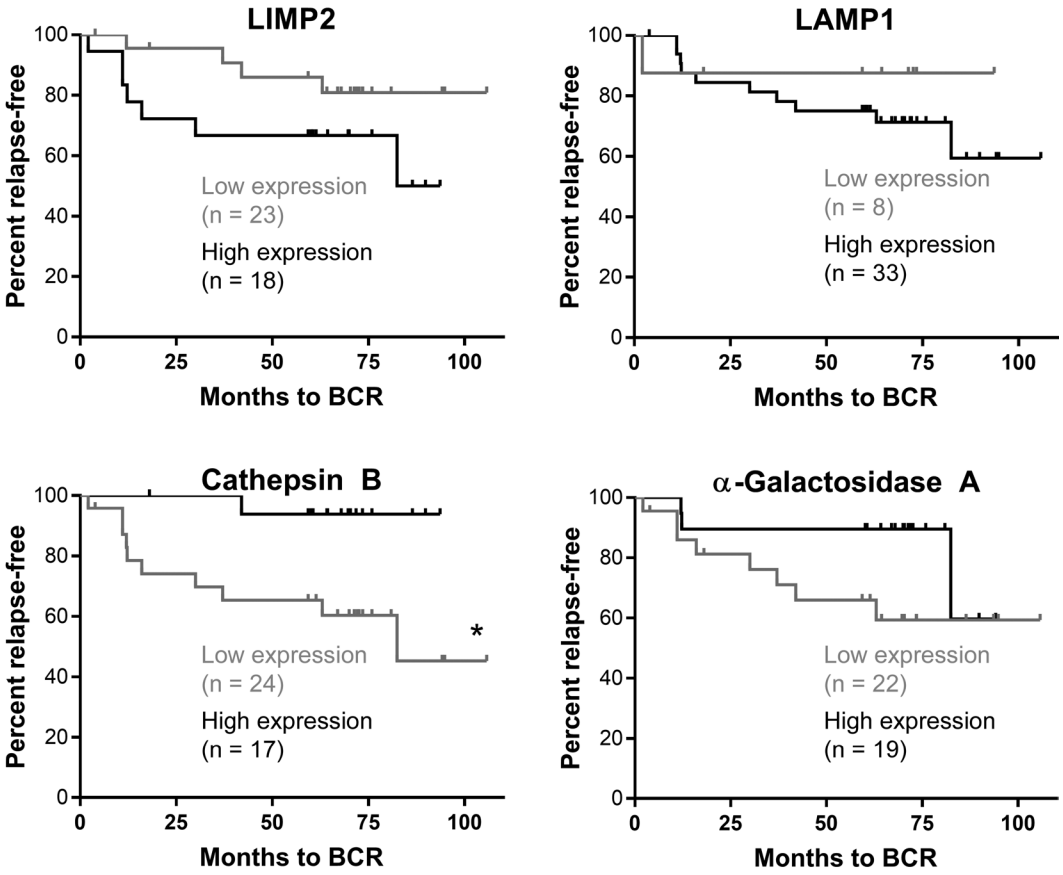
Patients expressing PSA protein greater than 7.9 ng/mL at the time of diagnosis had a significantly increased risk of biochemical recurrence when compared to the low-expression group (Hazard Ratio (HR) 2.204, $P = 0.0202$; Figure 5.8 A); however, there was a proportion of low pre-operative PSA patients (36.4 %) with biochemical recurrence at 106 months who would benefit from improved diagnostic and prognostic assays. Stratification of patients with PSA ≤ 7.8 ng/mL into high and low gene expression groups revealed a trend of high *LIMP2* expression to result in increased biochemical recurrence ($P = 0.118$; Figure 5.8 B). The expression of *LAMP1* did not appear to show any prognostic value. Clustering by cathepsin B expression indicated that patients in the low expression group were at significant risk of biochemical recurrence when compared to those in the high expression group (HR 0.0151, $P = 0.0151$; Figure 5.8 B). A trend was observed for the low expression of α-galactosidase A stratifying patients into a group with higher risk of biochemical recurrence (HR 0.3669, $P = 0.0705$; Figure 5.8 B).

Individual *RAB5A*, *APPL1* and *EEA1* gene expression was not able to stratify patients into statistically significant risk groups (Figure 5.9 A). However, stratification of patients based on the expression of a three-gene signature using *K*-means clustering methodology robustly separated patients into two groups with low or high expression of combined *RAB5A*, *APPL1* and *EEA1* (Figure 5.9 A). Kaplan-Meier survival analysis indicated patients in the high expression group of the three-gene signature were at significantly higher risk of biochemical recurrence when compared to those in the lower-expression group (HR 4.389, $P = 0.0303$, 95 % CI 1.344 – 15.38; Figure 5.9 B).

A



B

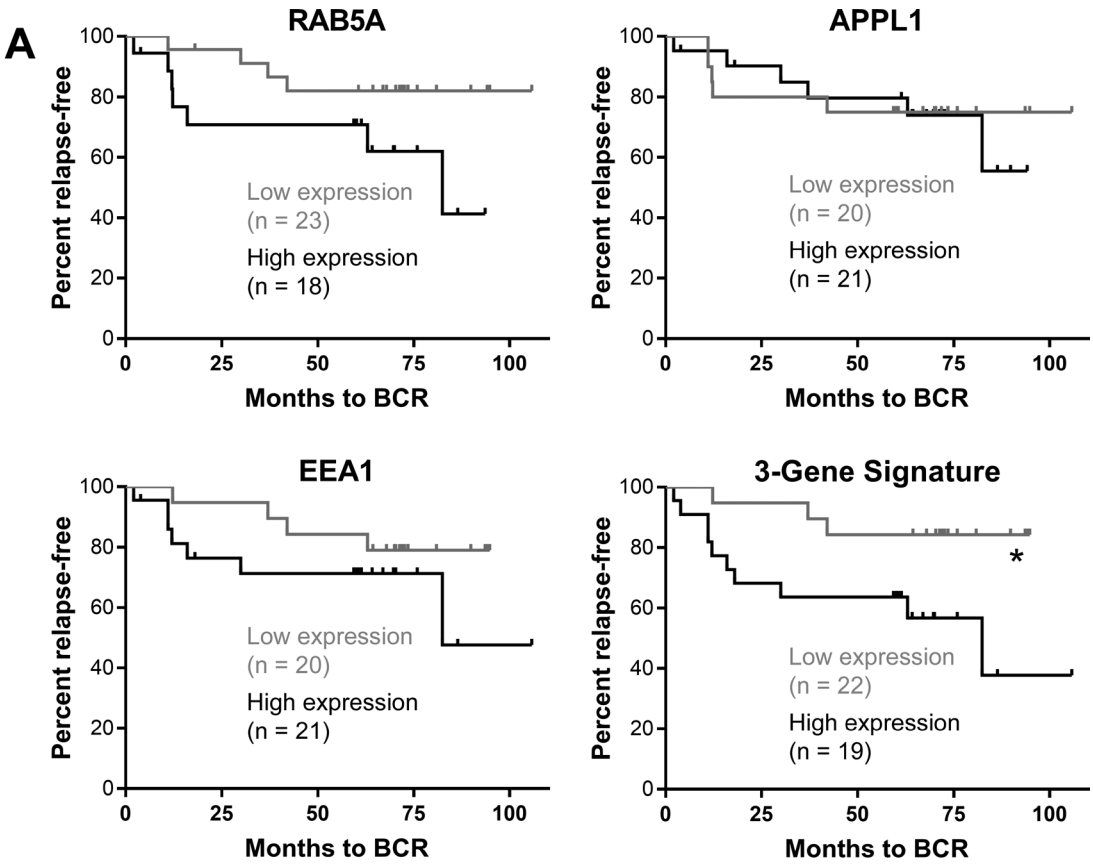


C

Gene	P	HR	95 % CI
LIMP2	0.1180	2.453	0.7588 - 8.344
LAMP1	0.5491	2.282	0.4113 - 8.590
Cathepsin B	0.0151	0.1151	0.0671 - 0.718
α-galactosidase A	0.0705	0.3669	0.1195 - 1.270

Figure 5.8 – Kaplan-Meier survival analysis of lysosomal gene expression for cancer patients expressing ≤ 7.8 ng/mL PSA.

Kaplan-Meier analysis of lysosomal gene expression showed significant capability for prognosis in patients expressing ≤ 7.8 ng/mL PSA based on cathepsin B expression. **(A)** Pre-operative PSA concentration of 7.8 ng/mL was used as a cut-off discrimination level for patient's stratification into poor- and good-prognosis subgroups. **(B)** From the good-prognosis subgroup of PSA ≤ 7.8 ng/mL, patients were further stratified into two groups by *K*-means clustering based on gene expression of *LIMP2*, *LAMP1*, cathepsin B or α-galactosidase A (high expression – black line, low expression – grey line). Statistical analysis was performed using Gehan-Breslow-Wilcoxon test. Statistical significance is marked by an asterisk (* $P \leq 0.05$). **(C)** Multivariate analysis of gene expression in prostate cancer recurrence. BCR: biochemical recurrence; G-B-W: Gehan-Breslow-Wilcoxon test; HR: hazard ratio; CI: confidence interval.



B

Gene	P	HR	95 % CI
RAB5A	0.1341	2.727	0.8432 - 9.504
APPL1	0.8708	1.115	0.3420 - 3.642
EEA1	0.1901	2.222	0.6811 - 7.365
3-Gene	0.0303	4.389	1.344 - 15.38

Figure 5.9 – Kaplan-Meier survival analysis of *RAB5A*, *APPL1* and *EEA1* expression and combined gene signature for cancer patients expressing ≤ 7.8 ng/mL PSA.

(A) Patients from the Glinsky cohort (Glinsky et al. 2004) expressing PSA ≤ 7.8 mg/mL were stratified into groups by *K*-means clustering based on *RAB5A*, *APPL1* and *EEA1* gene expression; the three-gene combined signature of *RAB5A*, *APPL1* and *EEA1* stratified patients based on BCR ($P \leq 0.0303$, Gehan-Breslow-Wilcoxon test; high expression – black line, low expression – grey line). **(B)** Multivariate analysis of gene expression in prostate cancer recurrence. BCR: biochemical recurrence; G-B-W: Gehan-Breslow-Wilcoxon test; HR: hazard ratio; CI: confidence interval.

5.3 Discussion

Endosome biology is altered in prostate cancer and this may promote cancer development and metastasis via elevated nutrient uptake or secretion of hydrolases that promote extracellular matrix degradation. Endosome-lysosome biogenesis is normally regulated by the transcription factor EB (Medina et al. 2011; Pena-Llopis & Brugarolas 2011), via control over the transcription factor of lysosomal genes containing "CLEAR elements" (Sardiello et al. 2009). Target genes of TFEB include *LIMP2* and *LAMP1*, which had differential expression in prostate cancer cell lines. Whilst we also observed elevated *LIMP2* and reduced *LAMP1* gene expression in prostate cancer tissue from these microarrays, there was no change in *TFEB* expression, suggesting that the normal action of TFEB (Sardiello et al. 2009) may be altered in prostate cancer.

Altered gene expression during cancer development may be a valuable prognostic indicator, and has been used in breast cancer, where increased expression of acid ceramidase in cancer tissue was associated with improved outcome (Ruckhaberle et al. 2009). Increased expression of acid ceramidase (*ASAH1*) may be effective for the diagnosis of pre-cancerous PIN lesions and may be associated with improved prognosis following intervention. Interestingly, the acid ceramidase gene maps to chromosome 8p22 that is frequently deleted in prostate cancer (Dong 2001; Seelan et al. 2000). The reduced expression of *ASAH1* observed in metastatic tissue compared to non-malignant tissue may be attributed to this deletion. Similarly, *CTSB* (cathepsin B), but not *CTSD* (cathepsin D), is found on chromosome 8p22. The downregulation in metastatic tissue of genes on chromosomal deletion points, as observed for *APPL1* (3p21), *APPL2* (12q24), *RAB5A* (3p24) and *EEA1* (12q22) and *RAB4A* (1q42) compared to their expression in non-malignant, PIN or primary cancer tissue may thus discriminate metastatic tissue and

provide an avenue for monitoring disease progression. The transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate cancer displays elevated gene expression in PIN tissue that is later reduced in tumours (Kela et al. 2009), suggesting that changes in the expression of lysosomal and endosomal genes observed in the Tomlins cohort may be involved in the initiation or promotion of pre-malignant cell survival, and that sustained expression is not essential for the continued function of the malignant cell. mRNA may be secreted from cells prior to, or post protein-synthesis (Liong et al. 2012; March-Villalba et al. 2012; Pelosi et al. 2006), which could affect the quantitation of gene and protein expression from tissue samples. Further study is required to determine if this affects tumourigenesis and whether gene and protein expression may be used for prognosis.

The variability in sample processing and inclusion of non-malignant tissue in cancer samples during processing of samples and RNA extraction for microarray analysis may alter the gene expression observed in prostate cancer compared with non-malignant tissue and mask potential candidates for the detection or monitoring of disease. RNA in the Taylor cohort was processed from samples by manually dissecting the tissue that contained greater than 70 % cancer cells (evaluated by a pathologist), whilst tissue from the Tomlins cohort was dissected using laser capture micro-dissection. Comparison of these two dissection methods has previously shown a striking difference in gene expression (Sugiyama et al. 2002), suggesting that the preparation of tissue samples remains a limiting factor in accurate gene expression analysis. Thus, the Taylor cohort does not provide certainty as to the type of cell harvested, and the heterogeneity of gene expression in cell subpopulations may lead to distorted gene expression (Butte 2002). Element-spotting of cDNA in the custom Tomlins microarray may also result in a less uniform and a less precise array than commercially available microchips (Katagiri & Glazebrook 2009). Ideally, the

same sample sets may be run on multiple microarrays and account for probe design differences, hybridisation techniques, and imaging and processing algorithm variations, increasing the consistency of results. This method of analysis is known to improve selection of genes that show only subtle differences in expression (Pedotti et al. 2008). Additional microarrays may be necessary for specific sets of genes, allowing for greater numbers of replicates on a single chip that increases the power of sample size for detection of small changes (Druyan, de Oliveira & Ashwell 2008). Due to these variables, further qRT-PCR analysis on patient samples is required. This data will definitively confirm or refute the altered gene expression we have observed in these microarray cohort studies.

Multivariate risk analysis and stratification of patients into low- and high-risk groups may provide a more individualised approach to prostate cancer detection and therapy, reducing over-diagnosis and over-treatment and thereby reducing patient morbidity and mortality (Vickers, Roobol & Lilja 2012). Active-surveillance is often used for patients deemed to have low-risk prostate cancer (e.g. clinical category T1c, Gleason score ≤ 6, and PSA ≤ 10 ng/mL), however older men are at an increased risk of mortality from prostate cancer despite these low scores and low PSA serum concentrations (Aizer et al. 2013). Examination of the survival curves of prostate cancer patients expressing low PSA, that represent a watchful-waiting group, reveals prostate cancers that are aggressive and result in more rapid recurrence (Glinsky et al. 2004), however additional factors such as Gleason score can improve patient stratification. Quantitation of a gene signature of the three endosome genes, *APPL1*, *EEA1* and *RAB5A*, in prostate cancer patients expressing PSA ≤ 7.8 ng/mL, led to patient stratification into high and low-risk recurrence groups. Further study will be needed to validate this methodology, to identify men at increased risk of developing life-threatening prostate cancer. The capacity of these endosomal genes

to stratify patients into prognostic risk groups suggested that they play an important role in the development and progression of prostate cancer and may therefore provide a new therapeutic target.

Chapter 6

Evaluation of endosome marker secretion for the detection of prostate cancer

6.1 Introduction

The primary serum marker that is currently used for prostate cancer diagnosis, is the prostate specific antigen (PSA)/human kallikrein 3 (hK3), but PSA does not adequately discriminate between cancer and benign pathologies nor differentiate aggressive and indolent cancers (De Nunzio et al. 2011; Hoffman 2011; Marta et al. 2013). More accurate and specific markers for prostate cancer diagnosis and prognosis are required to enable the implementation of early and appropriate treatment strategies. Early detection significantly reduces mortality for prostate cancer patients (Schroder, FH et al. 2012), making the development of accurate diagnostic and prognostic methods an important objective.

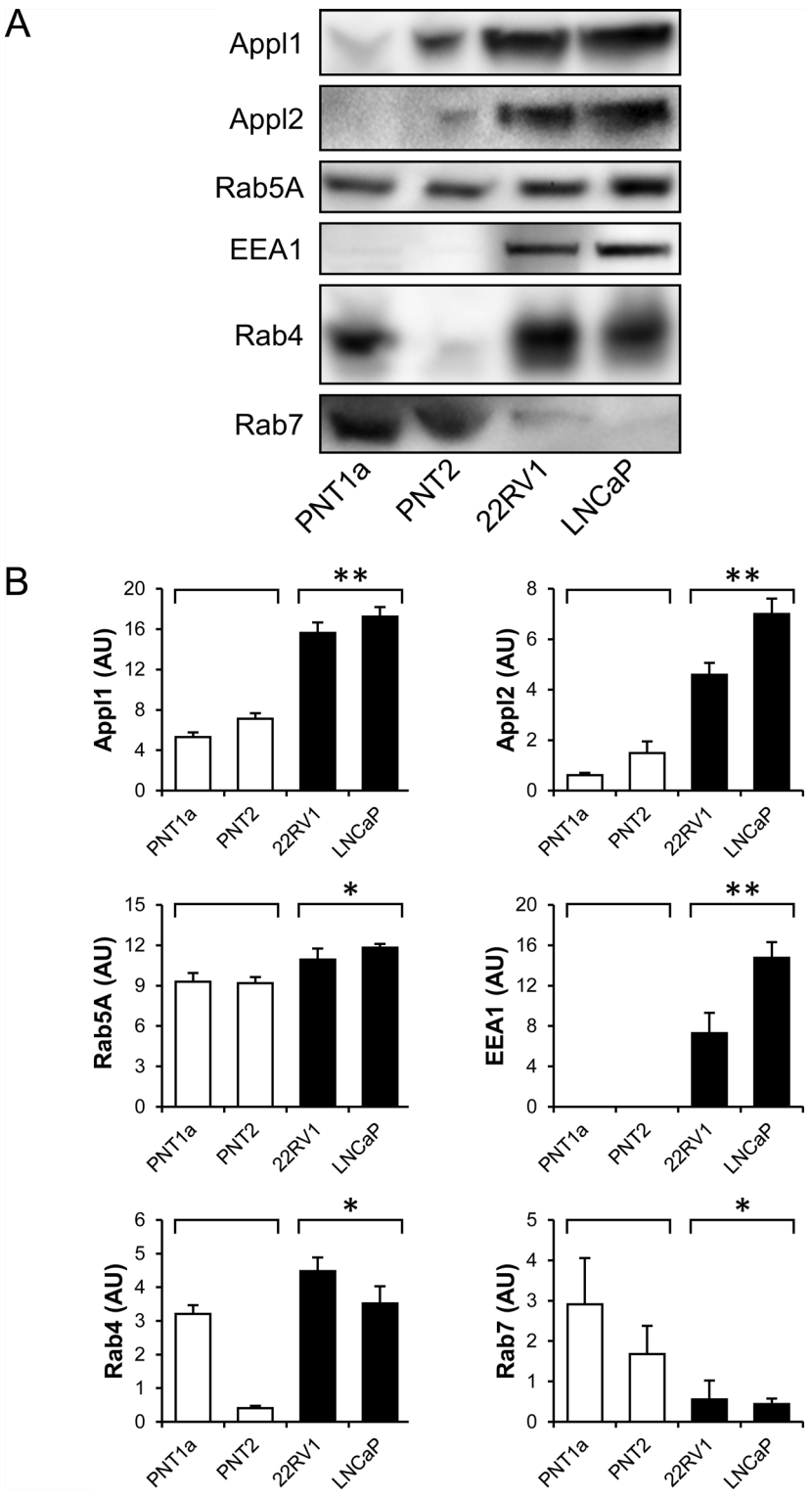
The ideal prostate cancer biomarker is one that is secreted in high amounts from the cancer and can be easily detected in patient biological fluids, such as blood and urine. Proteins that are secreted from cancer cell lines have been investigated as biomarkers and some have been validated *in vivo* (Villarreal et al. 2013). An analysis of the secretome from prostate cancer cell lines has been performed using two-dimensional electrophoresis and MALDI-TOF mass spectrometry (Sardana et al. 2008; Sardana, Marshall & Diamandis 2007), however, the underlying biology inducing the secretion of these biomarkers has not been analysed in depth. In addition, despite the discovery of a wide array of candidate protein biomarkers (reviewed Sardana, Dowell & Diamandis 2008), widespread clinical use of these markers has not yet come to fruition. Recently, caveolin-1 (Cav-1) has shown some promise as a biomarker for high-risk incidences of prostate cancer (Gumulec et al. 2012; Sugie et al. 2013). Caveolin-1 is found within microvesicles or exosomes that are secreted from the endosomes of prostate cells (Di Vizio et al. 2012; Parolini et al. 2009); and increased exosome release has been reported from various cancer cells (Cocucci, Racchetti & Meldolesi 2009). Rab GTPases such as Rab4, Rab5, and Rab7 are involved in exosome

function and release into the extracellular environment (Fevrier & Raposo 2004; Ostrowski et al. 2010; Savina, Vidal & Colombo 2002). The altered expression of endosome-associated markers that we have observed may contribute to disturbed exosome vesicle formation or secretion from prostate cancer cells. The secretion of the endosome vesicular machinery, identified from intracellular content analysed in chapter 4, has been analysed here and may identify appropriate biomarkers to facilitate the early detection of prostate cancer.

6.2 Results

6.2.1 Extracellular endosome proteins differentiate non-malignant and prostate cancer cell lines.

There was a significant increase in the amount of Appl1 and Appl2 detected in the culture media from prostate cancer compared to non-malignant control cell lines ($P \leq 0.01$). There was also a significant increase in the amount of Rab5A (approximately 20 %; $P \leq 0.05$) detected in the culture media from prostate cancer cell lines when compared to non-malignant controls. EEA1 was detected in the culture media from LNCaP and 22RV1 prostate cancer cell lines, but was not detected in the culture media from the non-malignant control cell lines PNT1a or PNT2 ($P \leq 0.01$). There was a significant increase in the amount of Rab4 detected in the culture media from prostate cancer compared to non-malignant control cell lines ($P \leq 0.05$), although the amount detected in the latter, PNT1a and PNT2 cell lines, was variable. There was a significant reduction in the amount of Rab7 detected in the culture media from the prostate cancer compared to the non-malignant control cell lines ($P \leq 0.05$).


A
Appl1
Appl2
Rab5A
EEA1
Rab4
Rab7
PNT1a
PNT2
22RV1
LNCaP
B
Appl1 (AU)
Appl2 (AU)
Rab5A (AU)
EEA1 (AU)
Rab4 (AU)
Rab7 (AU)
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*

Figure 6.1 – Detection and quantification of secreted endosome-related proteins from non-malignant control and prostate cancer cell lines.

(A) Representative Western blots of protein secreted from non-malignant control cell lines PNT1a and PNT2, and prostate cancer cell lines 22RV1 and LNCaP. **(B)** The amount of each protein in non-malignant control cells (white bars) and prostate cancer cells (black bars) was quantified by densitometry (arbitrary units) from triplicate Western blots (n = 3) and significant differences denoted by an asterisk (* $P \leq 0.05$; ** $P \leq 0.01$).

6.3 Discussion

The detection of biomarkers in prostate cancer patient serum could provide an effective method for patient diagnosis and prognosis. Currently the prostate specific antigen (PSA) is used as a diagnostic marker, but this has reliability issues and still requires an invasive biopsy to further determine the expected course for the cancer development. There is therefore an urgent need for new secreted biomarkers to enable accurate detection and prognosis of prostate cancer and to avoid unnecessary, invasive and costly procedures. We have observed significant increases in the amount of early endosome markers detected in the culture media from prostate cancer cell lines. In particular, the vesicular machinery associated with early endosomes, including Appl1, Appl2, Rab5A and EEA1 and recycling endosome protein Rab4, were markedly elevated in the culture media from prostate cancer cell lines, whilst the late endosome protein Rab7 showed a significant reduction in prostate cancer cells, when compared to non-malignant controls.

Rab GTPases such as Rab4, Rab5, and Rab7 are involved in exosome function and release into the extracellular environment (Fevrier & Raposo 2004; Ostrowski et al. 2010; Savina, Vidal & Colombo 2002). These Rab GTPases and other endosome markers such a EEA1 have been detected in exosome vesicles present, for example, in urine and released from colorectal cancer cells (Choi et al. 2007; Gonzales et al. 2009; Pisitkun, Shen & Knepper 2004). This suggested that the secretion of endosome markers observed from prostate cell lines may be of exosomal origin and not secreted as free protein into the extracellular environment. Exosome-containing multivesicular bodies (MVBs) can be distributed throughout the cytoplasm and cell periphery of metastatic prostate cancer cells, and this may enhance their exocytic release from the cell (Sahlen et al. 2004). Disturbances in MVB formation in late endosome compartments have previously been observed as a result of

disrupting endosome biogenesis (Fernandez-Borja et al. 1999; Llorente, de Marco & Alonso 2004) and resulted in altered Rab5-postive endosome morphology, reduced exosome formation and reduced secretion of caveolin-1 (Cav-1). Importantly, colocalisation of Cav-1 with the early endosome markers Rab5 and EEA1 (Aoki et al. 2007) suggested that exosome production can occur from early endosomes in addition to late endosome compartments. MVB biogenesis from the early endosome may therefore be promoted following the disruption of endosome biogenesis in prostate cancer. Consequently, the secretion of early endosome markers Appl1, Appl2 and EEA1, but not late endosome marker Rab7 from prostate cancer cell lines implied that the maturation of endosome compartments into multivesicular bodies and exosome vesicles is altered in prostate cancer and may therefore be used to distinguish prostate cancer from non-malignant tissue.

A possible mechanism by which exosome formation may be disturbed in prostate cancer involves the vesicular GTPase Rab5. The overexpression of Rab5 allows simultaneous exposure of PI3P and Appl binding domains on the Rab5 protein, which would effectively reduce Appl1 dissociation and enable concurrent binding of EEA1 (Zoncu et al. 2009). This could therefore result in endosomes that are Rab5-, EEA1- and Appl1-positive. Initiation of endosome membrane collapse and invagination to form exosomes is initiated by PI3P and the recruitment of Hrs and ESCRT complexes (Bache et al. 2003). The unusual composition of endosome vesicular machinery on prostate cancer endosomes may predispose exosome formation in early endosomes. The release of these exosomes from early endosomes would then account for the apparent secretion of early endosome vesicular machinery observed from the prostate cancer cell lines. This Rab5, EEA1 and

Appl1 secretion may provide the basis of an assay to enable the detection of prostate cancer.

Blood and urine are ideal patient samples for the detection of vesicular machinery secreted in exosomes, as they represent potential analytes for a non-invasive diagnostic assay. Indeed, cancer-derived exosomes have been detected in urine samples from patients with prostate cancer, with evidence of PSA-containing exosomes in urine (Mitchell et al. 2009) together with mRNA biomarkers within these exosomes (Nilsson et al. 2009). The urinary exosome protein database[15] provides evidence that Rab7, among other Rab GTPases, is normally detected in urine-derived exosomes (Pisitkun, Shen & Knepper 2004). Appl1, Appl2 and EEA1 proteins have also been characterised in association with urine-derived exosomes (Gonzales et al. 2009). In addition, the prostasome proteome has revealed Rab GTPases such as Rab 2, 3, 6 and 8 (Utleg et al. 2003), although Rab5 and other early endosome markers have not been reported. Prostasomes may normally be devoid of these early endosome markers. Further analysis of endosome biogenesis in prostate cancer cell lines is required to determine conclusively how the early endosome vesicular machinery is secreted. However, there is a precedence for Appl1 and Appl2 mRNA to be included within exosomes, as has been observed for colorectal cancer-derived exosomes (Hong, BS et al. 2009). This may contribute to the altered regulation, signalling and trafficking within cancer cells.

The altered secretion of early endosome and late endosome markers observed when comparing non-malignant control and prostate cancer cell lines provides a new avenue for investigation of diagnostic and prognostic markers that may lead to more accurate

[15] dir.nhlbi.nih.gov/papers/lkem/exosome/index.htm (accessed October 2012)

detection and prognosis of prostate cancer. The data here supports the notion that endosome biology is disturbed in prostate cancer, resulting in increased secretion of early-endosome markers and reduced secretion of late endosome markers from prostate cancer cell lines. Taken together, these results suggest that alterations in the secretion of early versus late endosome markers may provide the discrimination required for the early detection of prostate cancer.

Chapter 7

General discussion

7.1 Overview

This thesis investigated altered endosome and lysosome biology in prostate cancer *in vitro* and demonstrated significant changes in early endosome biology. This altered early endosome biogenesis was verified using microarray cohorts and other patient data. By contrasting the changes in early and late endosomes, a panel of biomarkers has been proposed with the potential to enable effective prostate cancer diagnosis and prognosis. There are a number of significant scientific implications for this biomarker discovery in terms of prostate cancer cell biology, which have been discussed below. The challenge arising from this new biomarker discovery is to translate this technology into clinical practice, to provide accurate, early detection and prognosis for prostate cancer patients.

7.2 Discussion

7.2.1 Altered endosome biology in prostate cancer

The endosome system is critical for the regulation of receptor-mediated signalling, cell metabolism, and cell proliferation, all of which are integrally involved in cancer biology. The biogenesis and maintenance of endosomal compartments is regulated by LIMP-2 (Kuronita et al. 2002; Kuronita et al. 2005) which here we have shown to be implicated in prostate cancer biology. LIMP-2 binds to the adhesive glycoprotein thrombospondin-1 (Crombie & Silverstein 1998), a ligand of CD36 that also shares homology with LIMP-2. Thrombospondin-1 is known to modulate cell adhesion and can potentiate the migration of cancer cells (Lahav 1993) as well as induce anti-angiogenic and anti-proliferative responses (reviewed by Sid et al. 2004). Interestingly, reduced expression of thrombospondin-1 has been observed in prostate cancer tissue and prostate cancer cell lines (Vallbo, Wang & Damber 2004). Moreover, increased cell surface expression of LIMP-2 can result in the uptake of thrombospondin-1 into endosomal compartments,

diminishing thrombospondin-1-mediated cell adhesion. This cell surface-expression of LIMP-2 may therefore affect tumour cell proliferation, adhesive potential and angiogenesis. The altered endosome biogenesis resulting from LIMP-2 overexpression may be directly involved in cancer development, and act to enhance cancer cell survival and metastasis. Recently, the activation of Rab5 has been implicated in the migration of normal and cancer cells and this promotes the disassembly of cell focal adhesions and enhances cell migration through phosphorylation and dephosphorylation of a focal adhesion kinase (Mendoza et al. 2013). Thus, altered endosome biogenesis may be inextricably linked to enhanced metastatic potential through the upregulation of critical endosome components that perform multiple regulatory functions. This suggests that aspects of altered endosome biology may be utilised to characterise the invasive potential of cancer cells and provide a method of both diagnosis and prognosis for the disease.

7.2.2 Implications for endosome signalling

Ligand-stimulated signalling is mediated and regulated by endosomes, and alternate trafficking of receptors in endosome sub-compartments may determine signalling outcomes (reviewed in Hupalowska & Miaczynska 2011; Miaczynska, Pelkmans & Zerial 2004). The endosome compartments are known to transduce mitogen-activated protein kinase (MAPK) and Akt pathways; and affect signalling through the trafficking of receptors to different endosome populations. This results in either amplified signalling through trafficking to the late endosomes (e.g. Epidermal Growth Factor Receptor), or sustained signalling via the recycling endosome. Altered trafficking of receptors such as the Epidermal Growth Factor Receptor may also be involved in the drug-resistance of cancer cells during chemotherapy (Wang, YN et al. 2010). Thus, endosome biogenesis and trafficking through endosome compartments plays a critical role in cell survival. Altered

signalling resulting from dysfunctional trafficking and endosome maturation may therefore promote cell survival in unfavourable physiological conditions, such as upon cancer cell metastasis.

Internal signalling pathways are regulated through the elimination of receptors and ligands, from the cytoplasmic environment, into internal vesicles of multivesicular bodies, targeting them for degradation and removing them from the intracellular signalling pathways. Intraluminal vesicles may also sequester signalling inhibitors, resulting in increased signal activation (Hupalowska & Miaczynska 2011), and thus be able to induce abnormal signalling in other cells, which may for example assist in angiogenic development and matrix remodelling (Fevrier & Raposo 2004). Disruption to the spatiotemporal location of multivesicular bodies, or reduced trafficking throughput of receptors, which were observed in prostate cancer cells, may therefore directly affect cell signalling and disrupt normal cell regulation. Signalling receptors such as Sonic hedgehog are essential for the regulation of cell proliferation and prostate gland development. The signalling pathways from cell-surface receptors that are involved in tissue development are also controlled by means of receptor endocytosis and trafficking through the endosome compartments to lysosomes, where the signalling potential of these receptors can be attenuated (Piddini & Vincent 2003). In normal development Sonic hedgehog signalling diminishes upon prostate maturity, however, the elevated expression of Sonic hedgehog has been correlated with metastatic cancer progression. Moreover, signalling cascades may be stimulated in non-malignant cells through the secretion of Sonic hedgehog from adjacent cancer cells, which may help promote multi-focal cancer development. The signalling cascades are mediated through endocytosis of the activated target molecule of Sonic hedgehog, Smoothened (Cohen 2010), and this endocytic process may be regulated

by the GTPase Rab23 that localises to endocytic vesicles (Evans, TM et al. 2003; Evans, TM et al. 2005). Thus, Rab23 may be involved in the trafficking of endocytic vesicles along microtubules, or the inactivation of Gli3, a repressor of Sonic hedgehog mediated signalling (Wang, Y, Ng & Tang 2006). Therefore, disruption of endocytic trafficking or recruitment of Rab23 to endocytic vesicles may promote dysfunctional signalling through the upregulated Sonic hedgehog pathway, enhancing cell proliferation and this requires further investigation.

7.2.3 Relation of altered endosome biology to altered cancer cell mitosis

Endosome-related Rab GTPases perform critical roles in cell function, and the disturbance of these Rabs may affect multiple systems, leading to cell deregulation and cancer genesis. Trafficking and adhesion control of the integrin receptor has been shown to be regulated by Rab21 (Pellinen et al. 2006) and this GTPase is also required for cytokinesis during mitosis (Pellinen et al. 2008). Moreover, abnormal trafficking of Rab21 is associated with aneuploidy that has been linked with cancer onset (Hognas et al. 2012; Sagona & Stenmark 2010). Cytokinesis also requires ESCRT machinery to sever the microtubules that connect two daughter cells (Mueller, Adell & Teis 2012). Endosome trafficking maintains the polarity of the cell (Mosesson, Mills & Yarden 2008), and this may also be disrupted in cancer cells, subsequently affecting the microtubule organising centre. We have hypothesised that early endosome exosome production and secretion is upregulated in prostate cancer, which may promote the favourable recruitment of ESCRT complexes to early, but not late endosomes, disrupting cytokinesis events that require these complexes. Alterations in endosome biogenesis in cancer cells may further affect the trafficking of vesicles delivering critical mitotic components to the intracellular bridge between two daughter cells, disrupting mitosis (Neto, Collins & Gould 2011; Schiel et al. 2012). It could

be speculated that the formation of tetraploid cancer cells may be a direct result of altered endosome biogenesis. Moreover, the prostate cancer cell lines 22RV1 and LNCaP have triploid chromosomes (Sobel & Sadar 2005b) that appears to mirror the chromosome profile of developing cancers *in vivo*.

7.2.4 Altered exosome genesis and secretion of endosome markers

The results presented in this thesis suggest a new phenomenon of enhanced exosome formation in early endosome compartments of prostate cancer cells and the release of early endosome vesicular machinery from these early endosome compartments into the extracellular environment. This phenomenon contrasts to the normal biology and secretion of exosome vesicles from the late endosomes of non-malignant cells (Keller et al. 2006). Altered exosome biology in prostate cancer cells may have a number of consequences in the initiation, development and progression of prostate cancer. A significant function of exosomes is the morphogenesis of cells and tissue, where the vesicles transport mRNA, miRNA and other critical intracellular components from one cell to another (Lakkaraju & Rodriguez-Boulan 2008). This may alter the polarity of cells that is critical for prostate cell differentiation and secretory function, as well as prostate tissue modelling (Pearson et al. 2008); the disruption of which may induce cancer onset (Lee & Vasioukhin 2008; Royer & Lu 2011). Exosomes may also induce altered intercellular signalling or inhibit signalling pathways that affects cancer development; thus, membrane-bound ligands on exosomes can be delivered to cell-surface receptors that activate signalling pathways and the endocytosis of exosome vesicles may be used to import functional protein and mRNA (Martins, Dias & Hainaut 2013). The biology of exosomes is partly regulated by the GTPase Rab11; the overexpression of which may result in the increased secretion of exosomes (Savina, Vidal & Colombo 2002). Interestingly, the results

by Savina et al. further demonstrated that Rab11 was critical for transferrin receptor trafficking and recycling. We have observed altered transferrin distribution in prostate cancer cells and this may have resulted from altered Rab11 expression in these cells, or disruption of the spatiotemporal location of exosome formation and the Rab11-mediated secretion of these vesicles.

Ubiquitinated proteins are recognised by the ESCRT machinery that induces membrane budding (Hurley 2008) and is directly involved in multivesicular body formation; however, the sorting of proteins may also occur in an ubiquitin-independent manner. Ubiquitin-independent protein sorting is mediated by ALG-2 (apoptosis-linked gene 2)-interacting protein X (ALIX), which has been shown to bind to the cytosolic domain of transferrin receptor via an internalisation motif (Geminard et al. 2004). ALIX induces the formation of exosomes through the recruitment of ESCRT-III (Baietti et al. 2012), and the population of exosomes in these multivesicular bodies consists of a distinctive proteome compared to exosomes derived from a ubiquitin-dependent pathway. Specifically, ALIX may not be dissociated from ligands upon recruitment of ESCRT-III, resulting in the incorporation of ALIX and ESCRT proteins into exosome vesicles (Hurley & Odorizzi 2012). The fusion of multivesicular bodies with lysosomes requires Rab7, however, this GTPase is also required for the release of ALIX-exosomes into the extracellular environment (Hurley & Odorizzi 2012). Interestingly, the scaffolding protein syndecan-1, which is known to recruit ALIX, showed altered cellular distribution in prostate cancer tissue and this biomarker was associated with an increased risk of mortality (Ledezma et al. 2011). Moreover, syndecan-1 showed significantly elevated expression in metastatic prostate cancer (Brimo et al. 2010). The altered cytoplasmic granular distribution and increased amount of syndecan-1 observed in metastatic cancer suggests that the biogenesis of multivesicular bodies may be

upregulated in prostate cancer, subsequent to the increased amount of protein that may undergo ubiquitin-independent sorting into exosomes. The quantitation of syndecan-1, ALIX, and other early and late endosome components such as the trafficking receptor sortilin, may reveal distinct populations of exosomes and differentiate between non-malignant and prostate cancer cells. A proteomic study of prostate-derived exosomes may provide a greater understanding into the altered endosome biology in prostate cancer, and the regulatory mechanisms and functions of secretion-related processes.

7.2.5 Significance and clinical translation

The results presented in this thesis indicate that endosome biogenesis is altered in prostate cancer and this may be used to differentiate between non-malignant and prostate cancer cells. The *in vitro* data has been further substantiated using microarray data and histology analysis of a selection of prostate cancer tissues. Further definition of the changes in endosomal biology in prostate cancer may be achieved through analysis of exosome vesicles, as these will provide an ideal source of biomarkers and have previously been demonstrated to have some potential for diagnosis in prostate cancer (Wayner et al. 2012).

Exosome vesicles and their content may provide an ideal method for prostate cancer testing because they can be sourced by non-invasive (urine) or semi-invasive (blood-serum) procedures. Prostate-derived exosomes have been detected in urine from prostate cancer patients (Nilsson et al. 2009), and biomarkers associated with these vesicles may be useful in diagnosis as well as monitoring treatment response (Mitchell et al. 2009). Whilst exosomes may be purified from blood or urine, the release of these vesicles into biofluids occurs from multiple cell types, therefore specific prostate markers, or a panel of markers showing differential expression that is unique to prostate cancer, are required. The specific

changes to late endosome derived exosomes and early endosome derived exosomes may provide this discrimination. In addition, exosomes have been shown to contain a "snapshot" of mRNA from cancer cells (Skog et al. 2008) and the transcript content is sufficient to quantitate gene expression of prostate cancer marker *PCA3* and *TMPRSS2* (Nilsson et al. 2009). The quantitation of early endosome related RNA transcripts in exosomes may therefore enable a novel method for patient risk stratification that avoids the necessity for tissue biopsies.

To facilitate the introduction of endosome related mRNA or proteins into a diagnostic biomarker panel it is essential to corroborate that changes in the endosomal biology of prostate cancer cell lines also occur *in vivo*. The development of *ex vivo* cell cultivation methods have enabled the growth and maintenance of human or mouse prostate tissue in standardised cell culture environments (Merchant 1990a, 1990b; Planz et al. 1999) that provide a source of prostate cancer cells exhibiting heterogeneity, an *in vivo* characteristic that is not available when using prostate cell lines. Cultivation of tissue as explants could also be used to facilitate the characterisation of altered endosome biogenesis and exosome release *ex vivo*. The development of immune assays that are specific to a panel of endosomal markers will provide data establishing the efficacy of endosome markers to detect and predict disease outcome in animals and humans. Prostate biopsies enable histological analysis using endosome markers, and a panel of endosome and lysosome markers will be validated on prostate tissue samples. Protein expression and distribution will be determined by immune-histochemistry in matched sets of human non-malignant and tumour prostate tissues. Significant and consistent differences in the amount of protein that are observed in these tissues will be further investigated in a cohort of prostate cancer progression tissue microarrays. This will allow the investigation of expression at the whole-

tissue and subcellular level, and how this changes during cancer progression. The primary endpoint for these studies will be endosome-related biomarker secretion into serum that can be quantified using either culture media from human tissue explants or patient samples. Specific immune assays will be developed to the critical endosomal markers that are altered in prostate cancer cell lines and their expression we will quantified in primary prostate cancer explants. The most promising markers or signature of markers can be assessed in matched tissue and blood samples from prostate cancer patients and men with benign prostatic hyperplasia and compared to age-matched controls. Analysis of additional endosomal genes in clinical microarray cohorts may identify genes for consideration in a biomarker panel. The gene expression data will be confirmed in non-malignant and prostate cancer tissue samples to generate and test a gene signature for cancer and or patient outcome. Two-dimensional RT-qPCR of tissue sections (Armani et al. 2011) may enable the more accurate quantification of these genes from prostate cancer and enable simultaneous comparison with non-malignant prostate tissue. An array of protein-based biomarkers also increases the specificity of disease diagnosis; serum concentration of IGFBP3 and CD14 proteins were elevated in advanced cancer and identified those prostate cancers that would undergo extracapsular extension (Oon et al. 2012). The specificity of the panel of candidate markers to prostate cancer should be determined so that markers that are unable to differentiate benign prostatic hyperplasia or prostatitis. These may further be assessed in cohorts with clinical outcome to test the capacity of the marker panel for prognosis, particularly when combined with Nonograms derived from these cohorts. The combination of a panel of endosome proteins and endosome related gene expression may provide a critical panel of biomarkers that when used in conjunction with current prostate cancer tests is able to improve their discriminatory ability. The altered endosome

biology of prostate cancer cells opens a new avenue for the investigation of therapies for prevention of the disease.

7.2.6 Conclusions

- HPV-18 transfected cell lines may not be the most optimal for prostate cancer biomarker discovery.
- The existing prostate cancer biomarkers and lysosomal proteins were unable to differentiate between a panel of non-malignant and prostate cancer cell lines.
- LIMP-2, a critical regulator of endosome biogenesis, showed increased expression in prostate cancer cells and is a potential new prostate cancer biomarker.
- Altered endosome traffic and function correlates with aberrant AKT signalling in prostate cancer cells.
- Secretion of early endosome machinery from prostate cancer cells may provide new biomarkers for patient diagnosis.
- Appl1 provides a new biomarker for prostate cancer histopathology.
- The expression of a panel of early endosome genes has the capacity to distinguish patients at risk of biochemical recurrence.
- There is altered endosome biogenesis in prostate cancer, with a specific change in early endosome biology and a reduction of late endosome trafficking and secretion (Figure 7.1). These seminal discoveries may provide avenues for the development of novel biomarkers for the diagnosis and prognosis of prostate cancer.

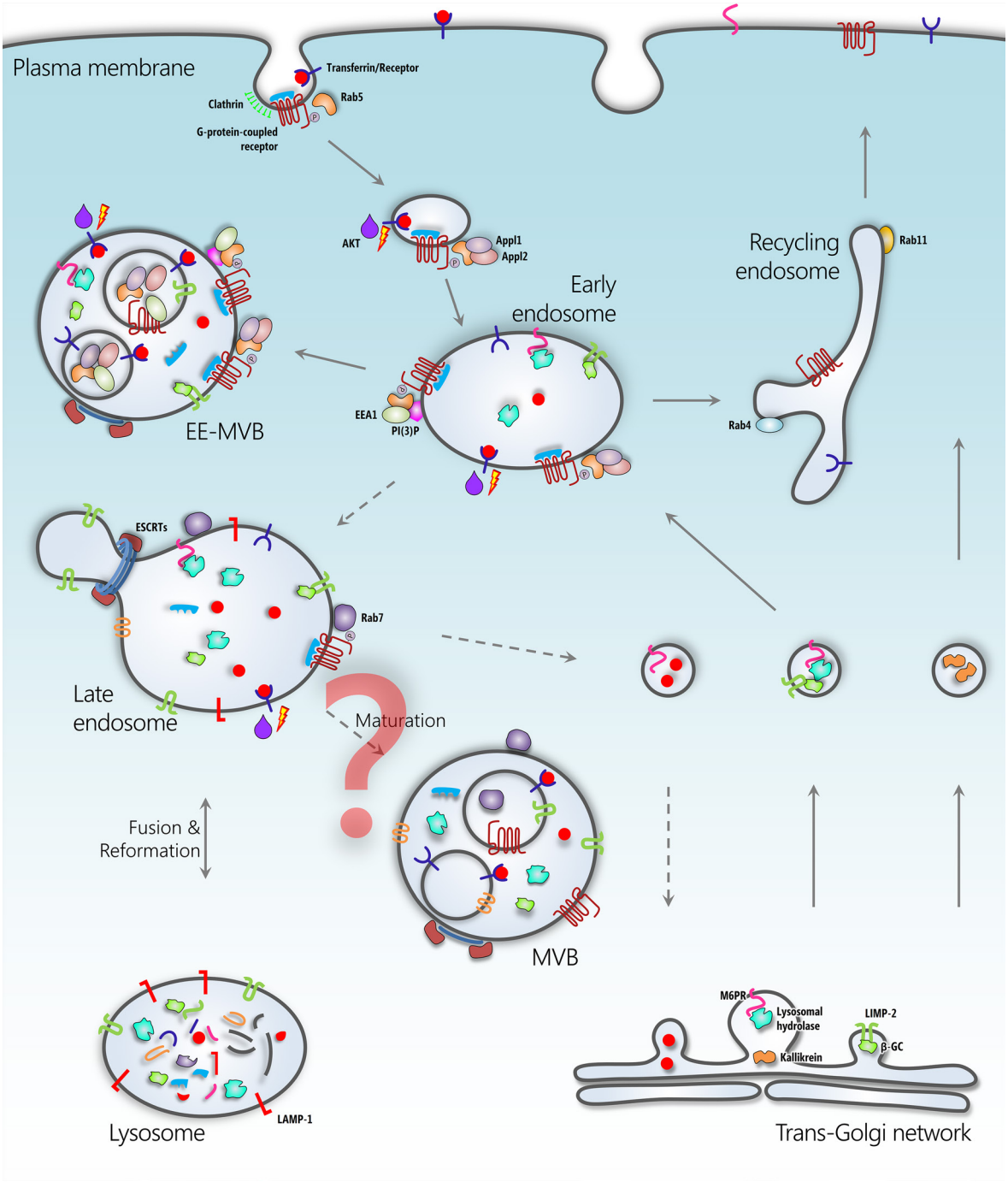

Figure 7.1 – Suggested model of altered endosome biogenesis in prostate cancer.
The formation of early endosome multivesicular bodies (EE-MVB) may occur preferentially in prostate cancer cells resulting in the release of early-endosome machinery into the extracellular environment. Maturation through the endosome system may by inhibited (dashed-arrows) resulting in aberrant signalling and altered receptor-ligand trafficking. Further characterisation of these changes may contribute to differentiating prostate cancer from non-malignant tissue.

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