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MOLECULAR FEATURES OF AGGRESSIVE PROSTATE CARCINOMA

SUBMITTED FOR THE DEGREE OF PHD IN HISTOPATHOLOGY AND MORBID ANATOMY

THE UNIVERSITY OF DUBLIN, TRINITY COLLEGE

LOUISE FLYNN

B.A. (HONS), M.SC.

2015

Declaration

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Table of Figures

List of Tables

List of Appendices

2.8 Full Seq Long Non-Coding RNA Lists

i. Summary

Prostate cancer is the most common form of malignancy in the male urinary tract and accounts for more than 20% of all newly diagnosed male cancer cases. The vast majority of prostate tumours are clinically insignificant, occurring in elderly patients who are unlikely to experience progression within their lifetime. However, rising incidence and mortality rates would testify that prostate cancer has the propensity to be an aggressive and fatal systemic malignancy. Despite the prevalence of this non-cutaneous malignancy, the pathobiology underlying the observed clinical heterogeneity of prostate cancer remains poorly delineated. This dearth of understanding has facilitated ineffective prognostication with overtreatment of insignificant disease and missed early intervention of the aggressive subtype emerging as significant clinical burdens. The perseverance of this clinical dilemma continues to frustrate the efficient management of prostate cancer, despite the widespread use of the somewhat contentious PSA (prostate specific antigen) screening measurement. Understanding the molecular underpinnings of aggressive prostate cancer is instrumental in order to effectively identify those high-risk patients and avoid needlessly invasive surgical intervention for those with indolent disease. Thus, there is an urgent and presently unmet clinical need for novel diagnostic and prognostic biomolecular markers in prostate cancer.

In recent years, technological advancements in expression profiling and genetic sequencing have deepened our knowledge of the molecular pathways whose dysregulation has long been implicated in the development and perpetuation of malignant cells. The quantification of ncRNA profiles in solid tumours has also revealed a regulatory substratum previously unappreciated. In addition, the paradigm of oncogenesis itself has been radically reviewed with the identification of discrete subsets of profoundly-proliferative self-renewing 'cancer stem cells' in haematopoietic and solid tumours. While the existence of a true 'cancer stem cell' is a controversial subject, there is an abundance of evidence to unequivocally substantiate functional plasticity amongst malignant cells. The functional mechanisms by which this cellular subpopulation pathologically influences tumour behaviour are ill-understood, largely owing to the non-existence of an efficacious *in vitro* model system to study malignant stem cells. Cancer stem cells are postulated to mediate treatment resistance and have been implicated in the perpetuation of malignancy and the development of distant metastasis, thus it is prudent to hypothesise that the cancer stem population may be associated with the predisposition of an aggressive or persistent disease phenotype.

In this thesis, the molecular characterisation of aggressive prostate cancer was explored through a large-scale expression analysis of a defined clinical radical prostatectomy cohort.

This project was undertaken as part of a collaborative effort by the Prostate Cancer Research Consortium to amalgamate transcriptomics, proteomics and pathological image analysis across multiple biological matrices in an attempt to identify a predictive panel of biomarkers. Furthermore, it was sought to develop a robust *in vitro* cancer stem cell model using cultured prostate cancer cell lines. Chapter 1 introduces the major clinical deficiencies in prostate cancer management and discusses the background and subsequent repercussions of the identification of cancer stem cells. Chapter 2 outlines the major techniques adopted throughout the course of this work including; real time PCR for gene and miRNA expression analysis, colony forming assay, high-salt agarose holoclone derivation, immunohistochemistry, murine xenotransplantation and ncRNA profiling using next-generation sequencing on the Illumina® HiSeq 2500 system. Chapter 3 describes the analysis of miRNA expression in a small archival cohort of 50 radical prostatectomy cases excised at St. James's Hospital and the subsequent immunohistochemical analysis of downstream protein expression in a tissue microarray of these cases.

Chapter 4 illustrates the integrated mRNA/miRNA expression analysis of a cohort of patients classified as indolent, significant or aggressive disease based upon a defined set of clinicopathological parameters. These samples were drawn from the dedicated Prostate Cancer Research Consortium bioresource having been collected across multiple institutions between 2006 and 2012. This chapter addresses some of the major confounding factors associated with the accurate quantification of expressional changes in solid tumour tissue and discusses the fundamental shortcomings of a study of this type.

Chapter 5 explores the limitations of a variety of cancer stem cell isolation techniques including flow cytometric sorting, high-salt agar holoclone generation, low-density culture in stem cell medium and colony forming assay. This chapter demonstrates the potential utility of long term cultured prostate cancer cell lines as a surrogate source of stem-like cells. As an adjunct to the genotypic characterisation of these cells, chapter 6 demonstrates the importance of substantiating cellular identity through murine xenotransplantation assays.

This thesis culminates in the interrogation of the molecular mechanisms of stem-like malignant prostate cells through the massively parallel sequencing of both small and long ncRNA repertoires of putative prostate cancer stem cells and their derivative xenograft tumours.

ii. Acknowledgements

This research was funded by the Irish Cancer Society and the Prostate Cancer Foundation. I would like to thank them for their continued support over the past three years.

I would also like to thank Prof. William Watson and all the members of the Prostate Cancer Research Consortium. I feel truly privileged to have been part of such a powerful collaborative research effort.

I would like to take this opportunity to thank my wonderful supervisors who have provided unwavering guidance throughout the course of this work. Professor Orla Sheils has been an unending source of support and inspiration, both professionally and personally over the past three years. Her kind words and interminable encouragement have allowed me to realise my full potential and for that I am incredibly grateful. I would like to extend my gratitude to Professor Stephen Finn for affording me the freedom to explore my own ideas. His positivity and confidence in my research provided me with renewed belief in my own abilities. I would also like to thank Professor John O' Leary for his continued enthusiasm and whose contribution has been instrumental to the development of this work.

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I owe my deepest gratitude to my father, Vincent, whose belief in me never falters and whose unconditional love and support has provided a constant source of reassurement.

Finally, I cannot find the words to truly express my gratitude to my mother, Alison. Her positivity and strength in the face of adversity has been an immeasurable source of inspiration for me. This thesis would not have been possible without her unwavering belief in my ability and for that I am forever grateful. This work is dedicated to you.

iii. Publications

University of Michigan, Cancer Centre Research Fall Symposium, October 2014: Retention of Stem Cell Characteristics in Prostate Cancer Cell Lines. Flynn L, Gallagher M, Ffrench B, Hams E, Fallon P, O' Leary J, Sheils O, Finn SP.

Prostate Cancer Foundation Annual Retreat October 2014: Retention of Stem Cell Characteristics in Prostate Cancer Cell Lines. Flynn L, Gallagher M, Ffrench B, Hams E, Fallon P, O' Leary J, Sheils O, Finn SP.

International Trinity Cancer Conference, Trinity College Dublin, September 2014: Retention of Stem Cell Characteristics in Prostate Cancer Cell Lines. Flynn L, Gallagher M, Ffrench B, Hams E, Fallon P, O' Leary J, Sheils O, Finn SP.

United States Academy of Pathologists Annual Conference 2014 (USCAP): Identification of Stem Cell Hierarchies in a Prostate Cancer Cell Line. Flynn L, Spillane C, O'Brien C, Sheils O, O'Leary JJ, Finn SP.

Irish Association of Cancer Research 2014 (IACR): Generation of Prostate Cancer Holoclones through Monoclonal Cultivation. Flynn L, Spillane C, O'Brien C, Sheils O, O'Leary JJ, Finn SP.

Trinity College School of Medicine Postgraduate Research Day 2013: Generation of Prostate Cancer Holoclones through Monoclonal Cultivation. Flynn L, Spillane C, O'Brien C, Sheils O, O'Leary JJ, Finn SP.

United States Academy of Pathologists Annual Conference 2013 (USCAP): miRNA Signatures of Indolent and Aggressive Prostate Cancer. Flynn L , Loda M, Watson W, Mucci LA, Sheils O, O' Leary JJ, Finn SP.

iv. Oral Presentations

"Retention of Stem Characteristics in Prostate Cancer Cell Lines"

Global Engagement of Doctoral Education Health Science Initiative, October 2014, Palmer Commons Forum Hall, University of Michigan, Ann Arbor, MI, USA.

"Generation of Prostate Cancer Holoclones through Monoclonal Cultivation"

6th Trinity School of Medicine Postgraduate Research Day, November 2013, Trinity Centre for Health Sciences, St. James's Hospital.

"Isolation and Characterisation of Prostate Cancer Stem Cells"

The Transdisciplinary Prostate Cancer Partnership (ToPCaP) Annual Meeting, May 2013, Fiskebäckskil, Sweden.

v. Awards

Awarded Best Poster Presentation at the International Trinity Cancer Conference, September 2014, Trinity Biomedical Sciences Institute; "Retention of Stem Cell Characteristics in Prostate Cancer Cell Lines"

Awarded Trinity College Research Continuation Scholarship 2014.

vi. Abbreviations

ABI	Applied Biosystems
AML	Acute Myeloid Leukaemia
AR	Androgen Receptor
ATCC	American Tissue Culture Collection
BCSC	Breast Cancer Stem Cell
BPH	Benign Prostatic Hyperplasia
BSA	Bovine Serum Albumin
CDK	Cyclin-dependent Kinase
CK	Cytokeratin
CML	Chronic Myeloid Leukaemia
CO_2	Carbon Dioxide
C_q	Quantification Cycle
CSC	Cancer Stem Cell
C_T	Cycle Threshold
CTC	Circulating Tumour Cell
DAVID	Database for Annotation Visualisation and Integrated Discovery
DNA	Deoxyribonucleic Acid
DRE	Digital Rectal Examination
EDTA	Ethylene-diamine tetra-acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMEM	Eagle's Minimum Essential Medium
EMT	Epithelial Mesenchymal Transition
EtOH	Ethanol
FACS	Fluorescent Activated Cell Sorting
FBS	Foetal Bovine Serum
FFPE	Formalin-fixed Paraffin-embedded
FGF	Fibroblast Growth Factor
H&E	Haematoxylin & Eosin
H_2O	Water
HGPIN	High-grade Prostatic Intraepithelial Neoplasia
HOXC	Homeobox C
HRPC	Hormone-refractory Prostate Cancer
HSC/HPC	Hepatic Stem Cell/Hepatic Progenitor Cell

HSPH	Harvard School of Public Health
hTERT	Human Telomerase Reverse Transcriptase
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IMS	Industrial Methylated Spirits
KDE	Kernel Density Estimation
KEGG	Kyoto Encylcopedia of Genes and Genomes
LAF	Laminar Air Flow
LCM	Laser Capture Microdissection
lincRNA	Larger Intergenic Non-coding RNA
lncRNA	Long Non-coding RNA
MET	Mesenchymal Epithelial Transition
miRNA	micro-RNA
mRNA	messenger RNA
MTI	miRNA-target Interaction
N_2	Nitrogen
NCRI	National Cancer Registry of Ireland
ncRNA	Non-coding RNA
NOC	Non Organ-confined
NOD/SCID	Non-obese Diabetic/Severe Combined Immunodeficient
OC	Organ-confined
PAP	Prostatic Acid Phosphatase
Pca	Prostate Cancer
PCRC	Prostate Cancer Research Consortium
PCSA	Prostate Cancer Stem Cell Antigen
PCSC	Prostate Cancer Stem Cell
PD-ECGF	Platelet-derived Endothelial Cell Growth Factor
PHS	Physician's Health Study
PIN	Prostatic Intraepithelial Neoplasia
piRNA	PIWI-interacting RNA
PRC	Polycomb Repressor Complex
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PSA	Prostate Specific Antigen

PSMA	Prostate-specific Membrane Antigen
RARP	Robotic-assisted Radical Prostatectomy
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
RP	Radical Prostatectomy
RQ	Relative Quantification
RRP	Radical Retropubic Prostatectomy
RT-PCR	Reverse-transcription Polymerase Chain Reaction
SCLGC	Stem Cell-like Glioma Cells
siRNA	Short-interfering RNA
snoRNA	Small Nucleolar RNA
SOP	Standard Operating Procedure
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
tiRNA	Transcription Initiation RNA
TRUS	Transrectal Ultrasonography
t-UCR	Transcribed Ultraconserved Regions
UGM	Urogenital Sinus Mesenchyme
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor

Introduction

Chapter 1

Chapter 1. Introduction

1.1 Prostate Cancer

Prostate cancer (PCa) represents a major cause of cancer-related mortality and morbidity in men, with an incidence higher than that of all other solid-organ malignancies (Abate-Shen et al., 2000). The molecular pathology of prostate cancer is complex; involving multiple genes and environmental factors. Older age, ethnicity and positive family history have long been recognized as significant etiological factors for the development of PCa. Prostate cancer is an inherently heterogeneous disease, which ranges in clinical behaviour from relatively indolent to rapidly fatal, aggressive malignancy (Hughes et al., 2005). Globally prostate cancer represents a significant health burden, although there exists a large variation in its incidence; the highest rates occurring in USA, Canada, Australia and France. In 2009, approximately 192,280 new cases of prostate cancer were recorded in the United States (Abate-Shen et al., 2000). In recent years, the mortality rates for prostate cancer have significantly declined in many developed countries (Damber et al., 2008). The clinical presentation of prostate cancer has also shifted, with a considerable increase in the number of men younger than 70 years of age being diagnosed with PCa. These temporal trends are consistent with the advent of prostate-specific antigen (PSA) measurement as a clinical screening test. However, PSA screening provides no prognostic information as it yields little or no insight into the biologic behaviour of a patient's prostate cancer. At present histopathological grading of transrectal ultrasonography (TRUS)-guided biopsy tissue by Gleason scoring is the best prognostic indicator in prostate cancer (Hughes et al., 2005).

The heterogeneous nature of prostate cancer complicates not only diagnosis but also management. The most common primary treatments for PCa are active surveillance (continuous monitoring by PSA testing and repeat TRUS-guided biopsy), surgical excision of the prostate (prostatectomy), interstitial brachytherapy (insertion of radioactive seeds), irradiation through external beam radiotherapy, and cryotherapy; each of which has associated caveats and significant post-operative morbidities (Niraula *et al.,* 2011). In the case of metastatic disease, these treatments are often supplemented with androgen-ablative therapy, which initially causes widespread regression of androgen-dependent malignant cells (Huggins *et al.,* 1941). However, it is almost inevitable that the tumour will re-emerge within 5 years resulting in a more aggressive and incurable hormone-refractory cancer (Damber *et al.,* 2008). Thus, at present there are several major clinical challenges, which hamper the effective diagnosis and treatment of prostate cancer. While it can be said that PSA testing has revolutionised the diagnosis of prostate cancer, in that it is now possible to detect prostate tumours at a very early stage, the early detection of prostate cancer needs to

be refined by improved biomarkers that can better stratify patients in conjunction with Gleason grading and also distinguish clinically indolent from aggressive prostate neoplasms. It is hoped that the examination of gene expression profiles, miRNA profiles, and cancer stem cells in prostate cancer will not only improve our current understanding of the mechanisms underlying prostate cancer but also yield novel biomarkers, which may allow for the development of non-invasive serum or urine tests for more accurate risk prediction in prostate cancer.

1.2 Epidemiology and Risk Factors

The incidence, mortality rates and to a lesser extent the prevalence of prostate cancer vary widely across the world. The intensity of screening initiatives and the differing availability of treatment are thought to have a major effect on disease incidence rates. The highest rates of prostate cancer incidence are observed in the United States, particularly among males of African American descent (Haas *et al.,* 2008). In contrast, China and Japan possess some of the lowest incidence rates. Among European countries, the most notable incidence rates are observed in Austria and France, where there exists a thorough prostate cancer screening program. In Ireland, prostate cancer represents the most frequently diagnosed cancer in men in 2013 (3014 cases) (National Cancer Registry Ireland, 2013).

The comparison of mortality trends with worldwide incidence figures has revealed that while large numbers of men are diagnosed with prostate cancer in the United States, few men die of this malignancy. In contrast, the vast majority of men who are diagnosed with prostate cancer in regions where incidence rates are generally lower, such as Asia, succumb to the disease (Stamey *et al.,* 2004). This finding indicates that the implementation of screening measures in developed countries may diagnose cancer at an earlier, more curable stage or it may infact be diagnosing more cases of biologically insignificant disease, which many posit creates unnecessary morbidity and cost.

The precise genetic and environmental determinants in prostate cancer have yet to be completely elucidated; however definitive risk factors include older age, ethnic origin and familial history of the disease. The probability of developing prostate cancer increases from 0.005% in men aged < 39 years to 13.7% among men aged between 60 and 79 (American Cancer Society, 2003). Autopsy data has revealed that prostate cancer is most prevalent in American men of African origin whose mortality rate is two to three times greater than Caucasian men (Crawford, 2003). The lowest global prevalence rates were observed in men of a Mediterranean and Japanese origin. The difference in rates observed between Caucasian men and men of African descent is likely due to a genetic predisposition to prostate cancer. However, the precise reasons behind the marked disparity between Eastern

and Western cultures are relatively unknown. Lifestyle characteristics associated with westernisation such as physical inactivity and high dietary fat intake have been postulated as putative risk factors (Brawley *et al.,* 1998). Studies have shown that countries in which dietary fat intake is higher display a concomitant increase in prostate cancer mortality rates. Furthermore, migration studies have shown that Japanese immigrants to the United States adopt higher rates of clinical incidence, which is highly suggestive that prostate cancer has an environmental influence (Crawford, 2003).

More than 85% of cases of prostate cancer are sporadic while the remaining 10-15% are subject to heritable genetic determinants (Carter *et al.,* 1992). The inability of linkage studies to conclusively identify highly penetrant inherited genes, which confer the prostate cancer phenotype, has been attributed to the possibility that genomic mutations in multiple low-penetrance genes are involved in prostate carcinogenesis. Although multiple putative susceptibility genes have been identified, the most important of which are *ELAC2, MSR1, CHEK2, NBS1* and *RNASEL*, the proportion of hereditary prostate carcinomas attributable to germline mutations in these loci is small (Hughes *et al.,* 2005). Studies have shown that the heritable form of prostate cancer accounts for a significant proportion of early onset disease. A segregation analysis of 691 families with a positive family history of prostate cancer has shown that 43% of cases are early-onset (<55 years) (Carter *et al.,* 1992). Furthermore, familial prostate cancer has been postulated to predispose a more aggressive form of the disease. A study by Kupelian *et al.* (1997) which compared the outcomes of patients with both hereditary and sporadic prostate cancer following radical prostatectomy, found that patients with the familial form of the disease have a much higher risk of biochemical failure. This result is highly suggestive that heritable prostate cancer has a much more biologically aggressive phenotype.

1.3 Screening

Prostate-specific antigen (PSA) is a serine protease produced exclusively by benign and malignant prostatic epithelial cells, which functions in seminal coagulation (Stenman *et al.,* 1999). It was first characterised in seminal fluid and subsequently in prostate tissue. PSA measurement, had for many years been used to monitor patients for disease recurrence until a number of studies demonstrated the efficacy of serum PSA measurement as a diagnostic marker for early disease (Carter *et al.*1992, Catalona *et al.* 1994). PSA measurement was introduced into clinical practise as a screening test in the 1990s and it remains a cornerstone for the early detection of prostate cancer (Barry *et al,* 2001). PSA is secreted into the blood by malignant cells via a disrupted basement membrane in tumour-affected areas of the prostate gland, thus an abnormally high PSA measurement is suggestive of the presence of

cancer (Simmons *et al.,* 2011). However, it is prudent to note that elevated PSA levels can occur as a result of other prostatic conditions, including benign prostatic hypertrophy (BPH) and prostatitis. The established upper limit of normal is 4.0 ng/ml, however only values greater than 10.0 ng/ml are thought to be truly indicative of cancer (Mettlin *et al.,* 1997). The calculation of percentage serum PSA that is free as opposed to bound is often used for greater discrimination in those patients who have BPH in addition to an elevated serum PSA reading. The percentage of free PSA is thought to be lower in cancer as PSA produced by malignant cells binds more avidly with serum proteins such as alpha-1 chymotrypsin and alpha-2 macroglobulin. Therefore, a low percentage of free PSA increases the likelihood that an elevated PSA reading indicates cancer (Simmons *et al.,* 2011). It is generally recommended that PSA testing be complemented by digital rectal examination (DRE) as studies have shown that PSA levels are within a normal range in 30% of cancers detected by DRE alone (Pentyala *et al.,* 2000). Furthermore, a US prevention study has shown that many men may still harbour prostate cancer despite low serum PSA levels (Thompson *et al.,* 2004).

The specificity of PSA value may be improved by several alternative PSA indices. They include PSA density (the PSA level divided by the prostate volume), PSA velocity (the rate of serum PSA increase over time) and PSA doubling time. While these modifications of serum PSA are capable of monitoring disease behaviour, they are rarely employed in clinical practise (Heidenreich *et al.,* 2013).

The widespread implementation of PSA screening measures in the early 1990s has had an undeniable impact on many epidemiologic features of prostate cancer including incidence, patient characteristics, treatment regimes, and disease outcome (Mettlin *et al.,* 2000). Data has shown that prostate cancer incidence rates have increased by 6.4% per year between 1983 and 1989, the average age at diagnosis has dropped and the proportion of cancers diagnosed at an advanced stage has also declined (Mettlin *et al.,* 1998). In the United States, the average age at diagnosis fell from 70.7 to 68.8 years between 1992 and 1995. Furthermore, the diagnosis of metastatic prostate tumours declined from 20.6 to 11.6% between 1986 and 1983 (Mettlin *et al.,* 1996), a trend also observed in Holland (Rietbergen *et al.,* 1999). In 1991, a significant decline in prostate cancer mortality began in the United States, although the significance of this data has been debated. Many postulate that the observed decline in mortality rates can be accounted for by the increased implementation of digital rectal examination and surgical intervention for organ-confined prostate cancer, which occurred in the decade preceeding the introduction of PSA screening (Mettlin *et al.,* 2000).

A recent study by Scosyrev *et al.,* (2012) has further analysed the effect of PSA screening on prostate cancer incidence trends through the estimation of the total number of patients who would be expected to present with metastatic prostate cancer in the modern US population if the age-specific and race-specific annual incidence rates of metastatic prostate cancer were equivalent to those prior to the introduction of PSA screening. The total number of patients who presented with metastatic prostate cancer in 2008 was computed and the number of cases, which would be expected to occur in the absence of PSA testing was estimated by multiplying each age-race-specific average annual incidence rate from the pre-PSA era (1983-1985) by the number of patients in the corresponding category in 2008 and adding the products. This study determined that if pre-PSA screening metastatic prostate cancer incidence rates were applied to the modern population, the total number of patients presenting with disseminated prostate cancer would be over three times greater than the number observed. These findings provide strength to the concept that PSA screening has altered the incidence of metastatic prostate carcinoma.

It has been argued that PSA testing may lead to the unnecessary treatment of patients with indolent, slow-growing disease who are unlikely to experience clinical progression during their lifetime. Moreover, for those with undetected metastatic spread, local treatment stemming from a PSA-based diagnosis would prove ineffective. It has been suggested that the rising incidence to mortality ratio in screened populations underscores the overtreatment of patients as a result of PSA testing (Klotz, 2005). While the evidence suggests that PSA testing has introduced a public health benefit, there remains no conclusive data to suggest the success of PSA screening as a diagnostic tool.

1.4 Sample Collection, Diagnosis and Grading

The standard method to obtain tissue samples for pathologic analysis and subsequent diagnosis of prostate cancer is a transperineal laterally-directed 18-gauge core biopsy. This procedure is clinically indicated on the basis of abnormal PSA level and/or DRE. Generally, TRUS-guided biopsy will focus on the outer peripheral zone and the apex of the prostate gland. Prophylactic oral or intravenous quinolone antibiotics are administered to the patient to minimise the risk of post-operative infection (Heidenreich *et al.,* 2011).

The Gleason grading system is the predominant technique for the histopathological grading of prostate adenocarcinoma in both needle-core biopsies and radical prostatectomy specimens (Humphrey *et al.,* 2004). This grading system was devised by an American pathologist, Dr. Donald F Gleason and is based on distinctive histologic patterns of malignant cells in H&E-stained prostate tissue sections. This technique defines five histological patterns with decreasing differentiation (1-5), which are used to obtain the

Gleason score by adding the primary grade and secondary grade pattern, i.e. the most prominent and the second-most prominent pattern (Gleason D.F., 1966) (Figure 1.1). In the case of only one grade being present upon microscopic inspection the Gleason score is obtained by doubling that grade. There is limited data on how to score carcinomas, which possess more than two grades. However, a study by Pan *et al.,* (2000) has highlighted the prognostic significance of high-grade tertiary Gleason patterns in prostatectomy specimens. This study reported that high-grade tertiary components i.e. Gleason pattern 4 or 5, which occupy <5% of the tumour volume have an effect on pathologic stage and progression rates, indicating that any tertiary components should be taken into consideration.

The most widely used clinical staging system for prostate cancer is the American Joint Committee on Cancer (AJCC) TNM system (Edge *et al.,* 2010). This system is predicated on five key parameters; the extent of the primary tumour (T category), the lymph node status (N category), whether distant metastasis is present (M category), the level of PSA at the time of diagnosis and finally, the Gleason score. There are four T categories, which describe the local extent of the primary tumour (T1-T4) and within these categories are a number of sub-categories. For example, T1 describes non-palpable disease while T2 describes palpable prostate carcinoma, which appears to be confined to the prostate. Within T2 there are three subcategories (a-b), which describe the localisation of carcinoma within the prostate gland. T3 describes prostate cancer, which has extended outside of the prostate perhaps to the seminal vesicles, while T4 describes the distant metastatic spread of cancer to other organs including the bladder and rectum. N categories are used to denote the status of lymph nodes, for example N1 indicates the presence of carcinoma in one or more nearby lymph nodes. Similarly, M categories indicate the presence/absence of distant metastatic lesions. M1 indicates that prostate cancer has spread beyond the lymph nodes and subcategories (a-c) indicate the precise localisation of metastatic disease. For example M1c, describes the presence of metastatic prostate cancer in organs such as lung, liver or brain. It is prudent to mention that the TNM system is updated periodically in accordance with advances in our understanding of prostate cancer biology.

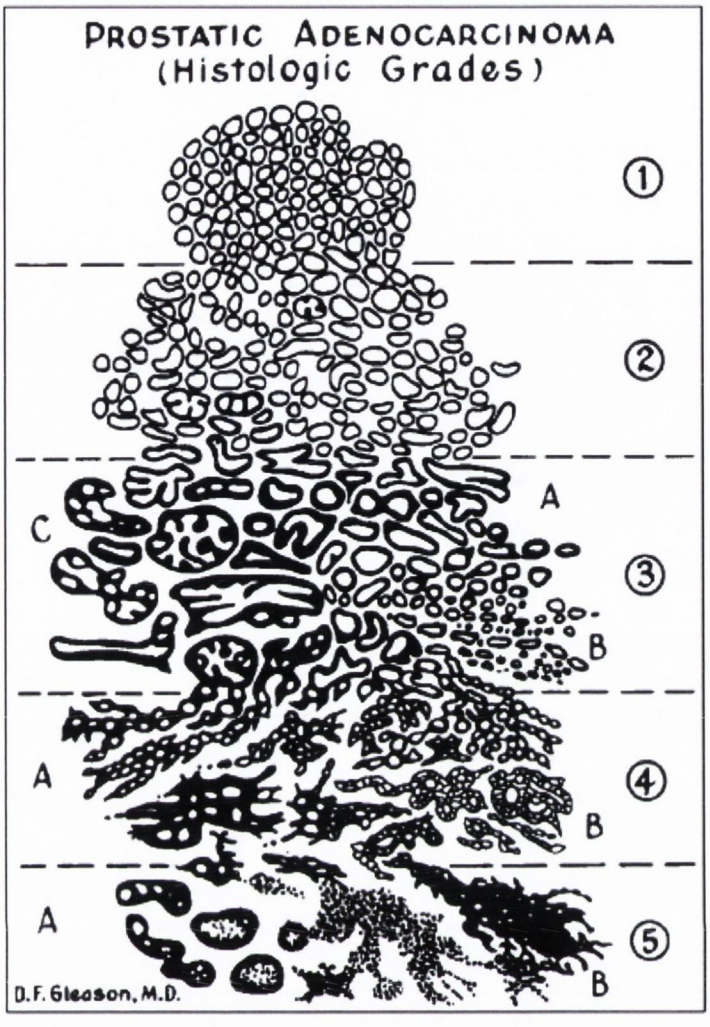

Figure 1.1 *Gleason Grading.*

Low Gleason grades are associated with small, more densely arranged cells. As the Gleason grade increases, cells lose glandular architecture. Gleason score is calculated by the summation of the two most prominent scores observed upon histopathological analysis. The conventional Gleason system was updated at a 2005 consensus conference of urological pathologists. The most important differences concern patterns 3 and 4. In the updated system poorly defined glands are classified in pattern 4, as is cribiform cancer (Epstein *et al.,* 2005). Adapted from Gleason D.F, (1966).

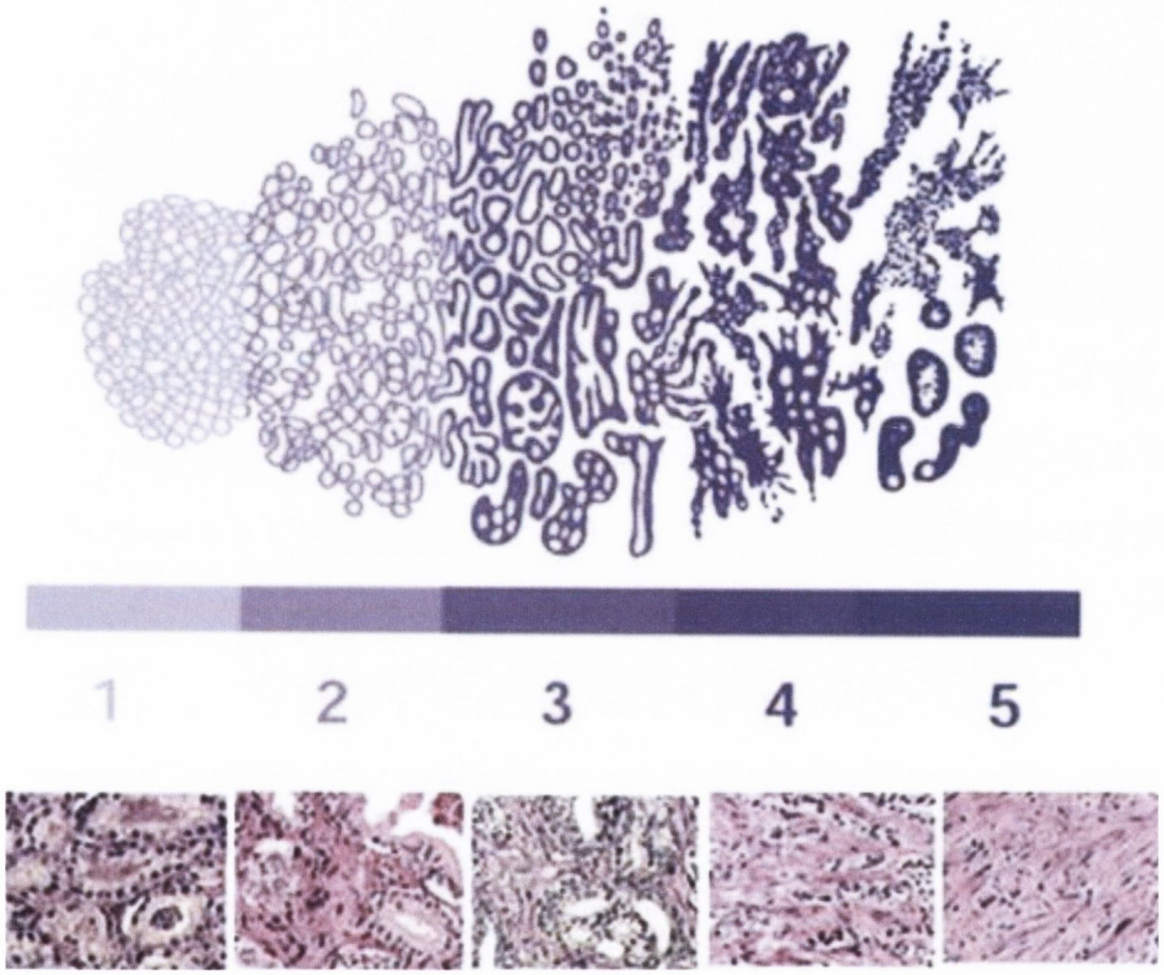

Figure 1.2 *Gleason Grading with H&E Equivalent.*

In grade 1, the cancerous prostate appears very similar to normal prostate. In grade 2, the glands appear larger with more areas of tissue between them. Grade 3 contains distinguishable glands, however at high magnification cells can be observed invading surrounding tissue. Grade 4 contains very little recognisable glands. Grade 5 demonstrates complete loss of glandular architecture. Adapted from Humphrey, 2004.

1.5 Treatment

In recent years, the clinical detection and treatment of prostate cancer has radically altered. As early as fifty years ago, patients diagnosed with metastases to bone and soft-tissue had a poor prognosis and died within 1-2 years (Denmeade *et al.,* 2002). However, during the past several decades there have been significant improvements in prostate cancer detection and surgical techniques for localised disease, which have led to the adoption of a more aggressive approach to the management of prostate cancer (Lu-Yao *et al.,* 1994).

1.5.1 Localised Prostate Cancer

The predominant treatment options for a patient with localised prostate cancer are active surveillance, radical prostatectomy or radiation. Active surveillance (AS) is acceptable in lieu of immediate treatment in patients with a low risk of progression (Bastian *et al.,* 2009). This strategy was devised with the fundamental aim of abrogating the overtreatment of patients with potentially clinically insignificant prostate cancer. Epstein *et al.* (1994) have defined the criteria for low-risk prostate cancer classification in needle-biopsy specimens as Gleason score ≤ 6, stage T1c, < 3 positive cores, < 50% cancer per core and PSA density < 0.15 ng/ml. These criteria were shown to be predictive of insignificant prostate cancer in 73% of cases. A further validation study demonstrated the ability of these criteria to identify pathologically insignificant prostate cancer in 94% of cases (Epstein *et al.,* 1998). Bastian *et al.* (2004) analysed the Epstein criteria in a 237-patient cohort who had undergone radical prostatectomy for prostate carcinoma defined as insignificant. The comparison of histopathological findings following RP with the Epstein criteria, demonstrated that 91.6% of patients did infact possess clinically indolent disease. However, it is prudent to note that the remaining 8.4% of cases, which had previously satisfied the Epstein criteria, were found to exhibit extraprostatic extension. While active surveillance may prevent the unnecessary immediate treatment of a proportion of patients, the associated caveat is that a small percentage of tumours, if left untreated may progress beyond the point of curative surgery.

The first radical perineal prostatectomy was performed by Hugh Hampton Young in 1904 (Young H. H., 1905). During the intervening years, prostatectomy was performed merely as palliative therapy to remove obstructive prostatic masses. This surgery was not employed with curative intent as the procedure was associated with significant post-operative morbidities including incontinence and loss of erectile function. Significant advances in surgical technique combined with new screening measures led to a dramatic increase in the number of patients treated with radical prostatectomy in the early 1980s. In 1983, Patrick Walsh devised a modified nerve-preserving approach, which allowed for the maintenance of sexual function in the majority of men with localised prostate cancer without compromising

the complete eradication of tumour cells. This technique avoids damage to the neurovascular bundles, which innervate the penis and also maintains urinary continence by preserving the nerves of the external sphincter and the inferior hypogastric plexus (IHP) (Pentyala *et al.,* 2000). Nerve-sparing prostatectomy surgery is believed to result in the absolute return of urinary continence in 98.3% of patients compared to 92.1% for standard prostatectomy (Hollabaugh *et al.,* 1998).

The first robotic-assisted radical prostatectomy (RARP) was performed in 2000 and this form of surgical procedure is rapidly gaining popularity among physicians and patients worldwide. Despite the lack of prospective randomised trials comparing this technique to other treatment options, RARP has become the predominant treatment for patients with localised prostate cancer in the United States. While there is no long-term follow up data for RARP in a large patient cohort, preliminary studies indicate that long-term patient outcomes are favourable (Badani *et al.,* 2007).

A post-operative increase in serum PSA is the first indicator of disease progression. Biochemical failure is defined as a serum PSA concentration of greater than 0.4 ng/ml, which rises continuously on two separate examinations (Stephenson *et al.,* 2006). Approximately 35% of patients will experience biochemical recurrence within 10 years following primary treatment (Han *et al.,* 2001). However, studies have demonstrated that the clinical course of disease following biochemical failure is protracted, with the median time from recurrence to metastasis being 8 years (Pound *et al.,* 1999). Vascular involvement and PSMA (prostate-specific membrane antigen) levels have been postulated as risk factors for disease recurrence. Studies have shown that PSMA levels remain elevated in patients who eventually experience biochemical failure, indicating its potential role in predicting treatment outcome. A short PSA doubling time has also been strongly associated with increased risk of disease progression and metastasis (D'Amico *et al.,* 2003). While PSA is used to detect pathological disease recurrence, Gleason grading of radical prostatectomy specimens remains the best predictor of progression following treatment. A study by Freedland *et al.* (2005) has demonstrated that the risk of prostate cancer-specific mortality is highest among patients with Gleason score 8 or 10.

1.5.2 Advanced Prostate Cancer

Huggins and Hodges were the first to demonstrate the dependence of prostate cancer cells on androgens, when they reported the palliative effect of testicular ablation in patients with metastatic prostate cancer (Huggins and Hodges, 1941). The suppression of androgen production through medical or surgical castration has since become the preferred treatment for patients with advanced prostate cancer. However, patients treated in this manner

inevitably progress to a more aggressive form of the disease known as hormone-refractory prostate cancer (HRPC). The development of resistant disease is thought to be associated with increased androgen receptor (AR) gene expression, which can occur through a number of alterations including AR gene amplification (Scher *et al.,* 2005). Chen *et al.* (2004) have shown that elevated AR expression was the most consistently observed alteration associated with HRPC in hormone-resistant xenograft models. Furthermore, they have shown that increased AR expression is necessary to develop resistance to androgen deprivation therapy in mouse xenograft models (Chen *et al.,* 2004). These findings are highly suggestive that elevated AR activity is a critical mechanism in the development of hormone-resistant prostate cancer.

However, it is prudent to note that alternative mechanisms for the development of androgen-refractory prostate cancer have been identified. These pathways include mutations within the androgen receptor and dysregulation of growth factors and cytokines. Mutations within the AR are believed to increase the number of ligands, which can activate the receptor resulting in the promiscuous activation of the androgen receptor through steroids and anti-androgens. While this molecular mechanism has been studied extensively, the proportion of hormone-refractory prostate cancers attributable to mutations within the androgen receptor is believed to be low (Debes *et al.,* 2004). Furthemore, alterations to the function or expression of growth factor coactivators such as insulin-like growth factor I have been demonstrated to result in the constitutive activation of the androgen receptor (Feldman *et al.,* 2001).

Hormone-refractory prostate cancer is characterised by rising prostate-specific antigen levels, progressive metastatic disease and worsening symptoms. Although, it is prudent to note that a subset of patients will exhibit progression to HRPC through a rise in PSA levels only (Chang, 2007). In more recent years, there has been a shift in the treatment of patients with hormone-resistant disease as novel therapeutics are developed. Secondary hormonal therapy has emerged as an important treatment strategy predicated on the finding that abrogation of androgen blockade in patients that have experienced disease progression can worsen patient symptoms (Fowler *et al.,* 1981; Taylor *et al.,* 1993). Furthermore, clinical trials incorporating PSA as a tumour marker have renewed interest in the use of cytotoxic chemotherapy in the management of HRPC. A trial conducted by Tannock *et al.* (1996) comparing the mitoxantrone/prednisone regime demonstrated that 29% of patients treated with this combination reported decreased pain compared with only 12% treated with prednisone alone. Thirty-three per cent of patients in the combination arm had a greater than 50% reduction in PSA-levels. In the 1990s, docetaxel emerged as a feasible and perhaps more clinically-effective treatment option for HRPC. A study by Petrylak *et al.* (1999) evaluating Docetaxel in combination with estramustine, demonstrated a higher rate of PSA

decline than mitoxantrone-based regimens. In more recent years, abiraterone acetate, an inhibitor of androgen biosynthesis has been demonstrated to prolong survival in metastatic castrate-resistant prostate cancer patients previously treated with chemotherapy (de Bono *et al.*, 2011). This hormone therapy is now widely administered in combination with prednisone for the treatment of disseminated prostate cancer. Despite these advancements in the treatment of hormone-refractory prostate cancer, its management continues to represent a clinical dilemma.

1.6 Diagnostic and Prognostic Biomarkers in PCa

The widespread use of PSA screening has undoubtedly improved prostate cancer detection as demonstrated by the large increase in the incidence of diagnosed prostate cancer since its implementation. However, this screening measure is largely believed to be responsible for the widely recognized problem of overtreatment (Stark *et al.*, 2009). The major clinical challenge associated with preventing overtreatment is the current inability to confidently distinguish at diagnosis indolent from aggressive cases of prostate cancer. While Gleason score and clinical stage are presently the strongest tools for the prediction of lethal prostate cancer and outcome following treatment, a panel of additional accurate biomarkers is required to determine which patients may be spared unnecessary and potentially harmful interventions. A better understanding of the molecular underpinnings of prostate cancer will undoubtedly help to achieve this.

To date, the identification of diagnostic and prognostic tissue biomarkers for prostate cancer has largely been based on immunohistochemistry, however a large proportion of putative prognostic markers have not been incorporated into clinical practice, likely owing to insufficient standardised methods to perform and interpret immunohistochemistry (Bjartell *et al.*, 2010). It is also possible that inadequate study design has played a role in the lack of clinically utilised biomarkers; specifically insufficient biomaterial and inappropriate end points. Many biomarker studies have yielded heterogeneous and at times contradictory results, which are considered largely inconclusive (Sutcliffe *et al.*, 2009). In recent years, biomarker research has advanced radically with the advent of high-throughput microarray technology, which has revolutionised our understanding of tumour biology. Expression profiling of mRNA and miRNA in particular, has emerged as a powerful tool for the construction of molecular signatures, which may indicate certain disease characteristics such as lethality or improve our ability to predict treatment response and outcome. It is hoped that the introduction of a robust panel of biomarkers into clinical usage will not only address the burden of overtreatment but also improve the quality of life and survival rates of prostate cancer patients.

1.6.1 Current Potentially Informative Diagnostic and Prognostic Biomarkers

AMACR

At present the diagnosis of prostate cancer is largely based upon the morphological features present within needle biopsy samples. In the majority of cases a confident diagnosis of prostate cancer can be made on morphology alone, however in those cases where the diagnosis of malignancy is inconclusive, immunostaining of biopsy specimens for alfa-methyl-CoA-racemase (*AMACR*) can convert the diagnosis from atypical to cancer (Magi-Galluzzi *et al.,* 2003). In recent years, microarray analysis has been used to demonstrate differential global gene expression between benign and malignant localized prostate cancer specimens and *AMACR* has consistently emerged as being overexpressed in prostate cancer (Luo *et al.,* 2001). A study by Rogers *et al.* (2004) demonstrated that the detection of *AMACR* in post-biopsy voided urine specimens was associated with the presence or absence of prostate cancer on biopsy in 21 of 28 patients examined in their study. Indeed, *AMACR* was detected in all 12 of the patients diagnosed with adenocarcinoma of the prostate. These findings indicate a basis for *AMACR* as a urine biomarker for the diagnosis of prostate cancer.

TMPRSS2-ERG

Chromosomal rearrangements involving members of the erythroblast transformation-specific (ETS) family of transcription factors have been identified in a large proportion of prostate carcinomas (Saramaki *et al.,* 2008). The most commonly observed of these being the fusion between the 5'-untranslated region of the androgen-responsive gene *TMPRSS2* and the ETS family member, *ERG.* It is noteworthy that gene fusions involving other members of the ETS family including *ETV1, ETV4* and *ETV5* constitute less than 10% of prostate cancer cases (Kumar-Sinha *et al.,* 2008). It is thought, given the substantial 3 Mb distance between *ERG* and *TMPRSS2* on chromosome 21q that the rearrangement occurs via either an interstitial deletion or an unbalanced interchromosomal translocation (Iljin *et al.,* 2006; Perner *et al.,* 2006). This fusion is thought to be present in approximately 50% of prostate cancers; however its prognostic significance remains controversial. Nam *et al.* (2007) have reported that the presence of *TMPRSS2-ERG* fusions is associated with increased risk of biochemical disease relapse, while Attard *et al.* (2008) reported that the high level of *ERG* expression as defined by the *TMPRSS2-ERG* fusion drives cancer progression and is associated with poor clinical outcome. Conversely, a recent study by Fine *et al.* (2010) reported that *TMPRSS2-ERG* translocation/deletion was not associated with the histological features of aggressive prostate cancer. Overall these findings point toward the *TMPRSS2-ERG* rearrangement being an early event in prostate cancer, which predisposes to clinical

progression of the disease. Despite the high incidence of these types of fusions in prostate cancer, the functional significance of the *TMPRSS2-ERG* rearrangement remains largely unknown. A recent study by Yu *et al.* (2010) found that *TMPRSS2-ERG* disrupts androgen receptor (AR) signalling by the inhibition of AR expression through epigenetic silencing, which profoundly disrupts the AR lineage-specific differentiation programme of the prostate. Furthermore, transgenic mice studies have suggested that *ETS* gene activation promotes epithelial mesenchymal transition (EMT) and therefore significantly correlates with a more aggressive disease phenotype (Wang *et al.,* 2008). Similar to previous studies, a recent study by Haggloff *et al.,* (2015) has demonstrated a strong association between the *TMPRSS2-ERG* fusion and poor clinical outcome. Interestingly, the presence of this fusion was found to be associated with a number of stromal factors including hyaluronan, Caveolin-1 and PDGFRβ. High expression of these stromal markers was found to be associated with poor outcome of prostate cancer patients. These findings suggest that there exists an interrelationship between the presence of TMPRSS2-ERG and stromal phenotypes specifically associated with poor prognosis. Overall, the *TMPRSS2-ERG* translocation represents a potential biomarker of clinical utility (Hessels *et al.,* 2007). This molecular event, if present in prostate-specific proteins within the urine, may reflect subtle changes within the prostate and act as a biomarker for the early diagnosis of prostate cancer.

It is noteworthy that while *TMPRSS2* has been identified as the only fusion partner of *ERG*, additional 5' partners have been identified for *ETV1, ETV4* and *ETV5* including *TMPRSS2, SLC45A3, HERV-K_22q11.23, C15orf21, CANT1* and *KLK2* (Kumar-Sinha *et al.,* 2008). It has been observed that ectopic overexpression of *ETV1* specifically is not sufficient to cause cell transformation; however overexpression of this gene drastically increases the invasive capacity of prostate cancer cell lines. Further *in vivo* studies utilising transgenic mice overexpressing *ETV1* in prostate epithelium have demonstrated that constitutive *ETV1* expression cannot induce tumour formation and that additional genetic lesions/environmental influences are required for the development of prostate carcinoma (Tomlins *et al.,* 2007). These findings indicate that preceding genetic anomalies facilitate dysregulated cellular proliferation, while *ETS* gene fusions, such as the aforementioned, stimulate the evolution to carcinoma (Kumar-Sinha *et al.,* 2008).

PTEN

PTEN is a critical tumour suppressor gene, which is frequently mutated or deleted in many cancers. The homozygous loss of *PTEN* results in activation of the phosphatidylinositol 3-kinase (PI3K) signal transduction pathway and is a common molecular event in advanced prostate carcinoma. It is interesting to note that while complete inactivation of *PTEN* is

observed in many cases of advanced prostate cancer, only one allele is lost in many patients at presentation. Thus, it has subsequently become clear that *PTEN* copy number has profound implications in prostate cancer. Analyses of *Pten* deletion in genetically engineered mouse models has demonstrated that prostate cancer incidence, latency and progression is directly associated with *Pten* dose within the prostate, suggesting that the hemizygous *PTEN* state is an early event, which plays a critical role in the initiation of prostate carcinogenesis (Trotman *et al.,* 2003). Inactivation of *Pten* has also been shown to cooperate with the *TMPRSS2-ERG* fusion (King *et al.,* 2009). It has been postulated that *PTEN* deletion and subsequent activation of PI3K pathway members predisposes to invasive adenocarcinoma, higher Gleason grade and androgen resistant disease (Wang *et al.,* 2003). These discoveries have led to the initiation of several phase I and II clinical trials examining the efficacy of targeting activated proteins in the PI3K pathway in prostate cancer patients (Morgan *et al.,* 2009). It could be hypothesised that the use of molecular biomarkers associated with this pathway may help to stratify patients who would benefit from such therapeutic regimens. Moreover, recent evidence has demonstrated that tumours with PTEN protein loss are more likely to be upgraded at radical prostatectomy than those with intact PTEN expression. Furthermore, PTEN loss in Gleason 6 biopsies identifies a specific subset of tumours, which are at increased risk of upgrading in the final prostatectomy specimen (Lotan *et al.,* 2015). These findings suggest that there may exist molecularly unique grades of Gleason pattern 3 prostate cancer, with PTEN protein loss marking an increased risk of occult pattern 4 presence. Evaluation of PTEN status in addition with other markers could aid in the identification of patients who are not suitable for active surveillance.

SPINK1/TAT1

Overexpression of tumour associated trypsin inhibitor (TAT1), which is also known as serine protease inhibitor Kazaltype 1 (SPINK1), has been consistently demonstrated in high grade prostate cancer (Paju *et al.,* 2007). A study by Tomlins *et al.* (2008) demonstrated that SPINK1 is exclusively expressed in *TMPRSS2-ERG* fusion-negative tumours and that SPINK1 is strongly associated with poor prognosis in patients treated with radical prostatectomy and is a general biomarker of aggressive prostate cancer. Additional support for the role of SPINK1 as a prognostic biomarker has come from a study by Leinonen *et al.* (2010), which examined a cohort of men with endocrine-treated prostate cancer and reported a significant association between SPINK1 overexpression and aggressive disease. A recent study, which examined the expression of TFF3, ERG and SPINK1 in a cohort of 279 radical prostatectomy specimens has demonstrated that an aggressive subgroup of prostate tumours coexpress TFF3 and SPINK1. Furthermore, SPINK1 positivity was found to be predictive of biochemical recurrence (Terry *et al.,* 2015). Although the reason behind the

significant correlation between SPINK1 overexpression and aggressive phenotype are unclear, it has been demonstrated that SPINK1 promotes proliferation of pancreatic cancer cells through activation of the epidermal growth factor receptor (Ozaki *et al.,* 2009). However, additional studies are required to fully understand the molecular mechanisms underpinning the involvement of SPINK1 in prostate cancer progression and its putative role as a prognostic indicator of aggressive disease.

NKX3.1

The homeobox gene *NKX3.1* plays a critical role in the normal differentiation of prostatic epithelium and downregulation of this gene is a frequent initiating event in prostate carcinogenesis (Abate-Shen *et al.,* 2008). *Nkx3.1* is the earliest marker for prostatic epithelium as it is expressed in all epithelial cells of infant prostate buds during development. *NKX3.1* is located on a 150Mb region of chromosome 8p21.2, which displays loss of heterozygosity in up to 85% of high-grade PIN (prostatic intraepithelial neoplasia) lesions and adenocarcinomas (Bethel *et al.,* 2006). This loss of expression has been shown to correlate with high Gleason grade, advanced tumour stage, metastasis and hormone-refractory disease (Bowen *et al.,* 2000). However there is controversy surrounding *NKX3.1* mRNA expression in prostate cancer as a number of studies have reported conflicting expression profiles for this gene. In particular, a study by Xu *et al.* (2000) has conversely demonstrated that *NKX3.1* overexpression correlates with metastatic disease. Whereas, other studies have indicated that *NKX3.1* mRNA is expressed at similar levels in both normal prostate tissue and tumours of varying grades (Ornstein *et al.,* 2001; Korkmaz *et al.,* 2004). While it is clear that *NKX3.1* represents a haploinsufficient tumour suppressor gene, which is implicated in the initiation of prostate carcinogenesis, additional studies are required in order to elucidate how *NKX3.1* may be utilised clinically as a tissue biomarker for prostate cancer.

1.6.2 mRNA Expression Signatures

The development of microarray-based technology has allowed for the global characterisation of gene expression profiles that molecularly characterise prostatic neoplasms. It is hoped that this knowledge may not only assist in the identification of genes, which anticipate the clinical behaviour of the disease but also the identification of clinically feasible biomarkers to better decision making in the management of prostate cancer. Many large-scale gene expression studies examining prostate cancers of different grade and stage have been performed (Dhanasekaran *et al.,* 2001; Luo *et al.,* 2001; Welsh *et al.,* 2001; Lapointe *et al.,* 2004; Singh *et al.,* 2002). While these studies have identified global gene expression differences, which are capable of distinguishing normal from tumour prostate tissue and identifying metastatic disease, the majority of these signatures are not sufficiently accurate

to replace morphology-based diagnostics and prognostics. These gene expression studies remain significant in the setting of biomarker discovery but their translation into clinical utility is hampered by a number of complications. In particular, most of these studies have used arrays containing up to 35,000 genes and it is likely that much smaller probe sets would be used in a clinical setting. Furthermore, the proportion of each specimen analyzed was often limited as these studies required frozen material. In addition, the wide variation which exists in results between studies has raised concern over the reliability of microarray expression profiling. Much of this variation is likely due to the use of different microarray platforms, differences in sample preparation protocols and the variability of sample size across studies.

However, not all of these studies are insignificant with regards to potential clinical utility. An important study by Nakagawa *et al.* (2008) has identified a tissue biomarker panel, which predicts the systemic progression of prostate cancer following PSA recurrence. As previously mentioned, the majority of men diagnosed with low-risk, organ-confined prostate cancer are treated with radical prostatectomy (RP), interstitial brachytherapy or external beam radiation. The average biochemical PSA recurrence rate at 5 years post-primary treatment is 6% for low-risk patients (Simmons *et al.,* 2011). This represents a heterogeneous cohort of patients, some of which will have local recurrence or develop metastasis followed by hormone refractory disease; however the majority will have no other evidence of disease progression apart from the relapse in PSA levels. It is prudent to note, that PSA elevation following radical prostatectomy or radiation therapy is associated with a 15% to 25% five year prostate cancer death rate. Nakagawa *et al.* (2008) hypothesised that additional markers may aid in the identification of men with an elevated PSA following radical prostatectomy who will likely suffer systemic progression and thus require additional treatment. This study employed a microarray expression analysis of 1021 cancer-related genes in order to identify those genes specifically associated with outcome in a cohort of men who developed systemic progression within 5 years of PSA recurrence. A matched control group of men who exhibited elevated PSA levels within 5 years following radical prostatectomy with no other evidence of clinical progression was also included in the study. This study demonstrated that the resultant 17-gene systemic progression model generated an AUC of 0.88 and performed significantly better than clinical variables alone. In addition to this 17-gene panel, Nakagawa *et al.,* (2008) tested the power of a number of previously reported prognostic gene expression signatures for the prediction of systemic progression and PSA recurrence in their patient cohort. In most cases the predictive models correlated well. In particular a 5-gene signature developed by Singh *et al.,* (2002), a 23-gene signature from Lapointe *et al.,* (2004) and a 70-gene signature from Yu *et al.,* (2004) yielded AUCs similar to that of the 17-gene predictive panel. This study indicates that the analysis of gene

expression patterns may help to identify patients who would benefit from further therapy following a rise in PSA levels, thereby preventing unnecessary treatment.

Another study of significant prognostic value in prostate cancer is that by Ramaswamy *et al.*, (2003). In an attempt to elucidate the molecular underpinnings of metastasis, a microarray expression analysis of several diverse tumour types (lung, prostate, breast, colorectal, uterine and ovarian cancer) was performed. This study identified a gene expression signature capable of differentiating primary from metastatic adenocarcinomas across the tumour types. It was also noted that a small subset of primary tumours expressed a similar gene profile to metastatic tumours, leading to the hypothesis that a 'metastasis programme' may already be present in the bulk primary tumour at the time of diagnosis. Moreover, the final 17-gene metastasis signature was found to be strongly associated with a poor clinical outcome in prostate cancer. The widely accepted notion is that the propensity of a tumour to metastasise depends on a rare subpopulation of cells within the primary tumour; however the findings of this study give weight to the argument that the clinical behaviour of a tumour and overall patient outcome can be predicted from profiling of the primary bulk tumour.

A more recent study by Penney *et al.* (2011) has employed a similar approach in an attempt to construct a molecular signature of lethal prostate cancer. This study developed an mRNA signature capable of differentiating high from low Gleason scores in two large, well-characterised prostate cancer cohorts; the Swedish Watchful Waiting Cohort (Andren *et al.*, 2006), and the Physicians Health Study (PHS) (1989). It was then examined whether the 157-gene signature could improve the prediction of lethal prostate cancer in men with a Gleason score of 7. This signature was found to be statistically significantly predictive of lethal disease among patients with Gleason score 7, irrespective of 3 + 4 or 4 + 3 status. Furthermore, a marked homogeneity in expression of this gene panel was identified among patients with Gleason score ≤ 6. Conversely, a consistent diversification in expression of this signature was observed among those patients with a higher Gleason score (≥ 8). These results are consistent with previous findings, which report that there exists a direct correlation between molecular phenotype and histological classification (True *et al.*, 2006). This study in particular, has immense clinical significance as the majority of prostate cancer patients present with seemingly low-risk disease and intermediate Gleason scores of 6 or 7. Thus, there is an urgent need to improve risk prediction in this particularly heterogeneous group of patients, in order to avoid unnecessary treatment.

The limited predictive capacity of currently employed diagnostic and predictive indicators in prostate cancer underscores the fundamental need for clinically feasible novel biomarkers. While microarray expression studies have yielded an abundance of significant data and

facilitated the translation of a number of diagnostic markers including *AMACR* into a clinical setting, major inconsistencies between studies hamper the clinical utilisation of gene signatures. These inconsistencies are largely owing to the use of differing platforms and the lack of standardised methods for sample extraction and preparation. The heterogeneous nature of prostate cancer is also likely to complicate precise molecular profiling, as many studies employ relatively small sample sizes, resulting in bias. A mathematical model devised by Ein-Dor *et al.,* (2006) has proposed that a typical breast cancer gene expression profiling study would require several thousand patients in order to achieve an overlap of 50% between two predictive gene lists. While much has already been achieved in this field, there remains an unmet need for more powerful diagnostic and prognostic biomarkers in prostate cancer.

1.7 miRNAs and Prostate Cancer

microRNAs (miRNAs) are a small, endogenously expressed, non-coding RNA species of 18-25 nucleotides in length, whose critical role in gene regulation was first described in seminal work by Victor Ambros and colleagues (Lee *et al.,* 1993). They discovered that *lin-4,* a gene known to regulate developmental events in *C. elegans* does not encode a protein, but rather generates a pair of small RNAs of 22 nt and 61 nt in length (Lee *et al.,* 1993). The shorter 22 nt *lin-4* RNA was to become the founding member of a profoundly important class of small, regulatory RNAs known as miRNAs. miRNAs have since been shown to negatively regulate target protein expression through translational inhibition or mRNA degradation (Bartel, 2004). miRNAs are now known to be widely involved in the coordination of many physiological processes including proliferation, differentiation and apoptosis through the repression of thousands of target genes at the translational level. Furthermore, aberrant miRNA expression has been implicated in a number of human pathologies, including cancer. Emerging data has supported the concept that miRNA expression patterns can be easily detected and harnessed for not only the classification of cancers but also the prediction of cancer behaviour and outcome.

1.7.1 miRNA Biogenesis and Function

Most miRNAs are believed to derive from independent transcriptional units, however approximately one quarter of human miRNAs reside within the introns of pre-mRNAs, which suggests that these miRNAs are not transcribed from their own promoters but rather share regulatory elements with their host genes (Aravin *et al.,* 2003). This arrangement implies the coordinated expression of a miRNA and a protein, a favourable mechanism given the regulatory function of miRNAs. miRNA biogenesis begins with the transcription of large, often 1kb, primary miRNA (pri-miRNA) transcripts by RNA Polymerase II (Figure 1.3). The

miRNA precursor (pre-miRNA) is a 60-70nt stem loop structure, generated when the pri-miRNA is cleaved by an RNase III endonuclease known as Drosha (Lee *et al.*, 2003) (Figure 1.3). The pre-miRNA is transported from the nucleus to the cytoplasm by Ran-GTP and Exportin-5 (Yi *et al.*, 2003). A further cleavage event by the enzyme Dicer, which removes the stem loop of the pre-miRNA, generates a transient duplex comprising the mature miRNA and a remaining fragment of the pre-miRNA known as the miRNA* sequences (Lau *et al.*, 2001). The miRNA strand of this duplex becomes incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC), and the miRNA* is removed and degraded. Studies have indicated that the specificity of the strand which enters the RISC is based largely on the stability of the duplex, as the 5' end of the strand that enters RISC is generally less tightly paired (Khvorova *et al.*, 2003). These findings suggest that a helicase-like enzyme samples each end of the duplex multiple times before proceeding with progressive unwinding, which results in a bias for unwinding of the duplex at the more accessible end.

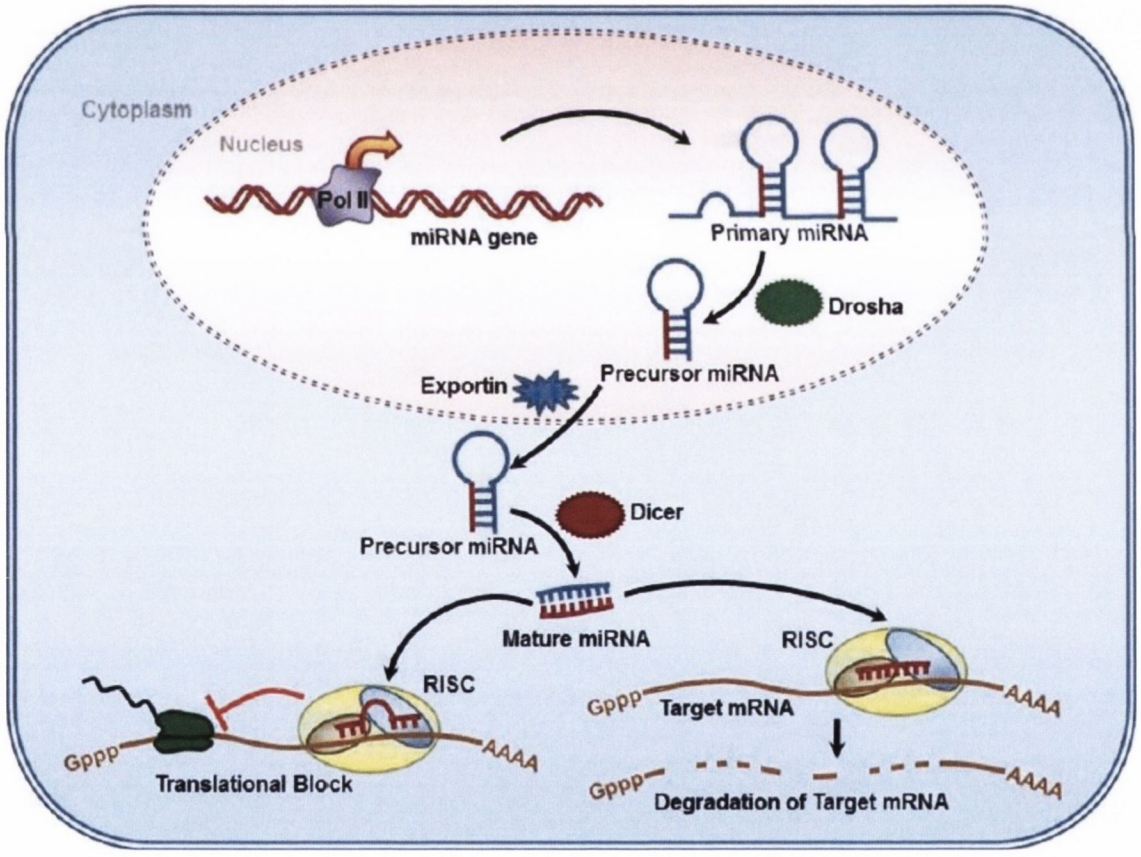

Figure 1.3 *miRNA Biogenesis and Post-Transcriptional Regulation.*

Adapted from Joshi et al., (2011).

miRNAs orchestrate posttranscriptional regulation by either of two mechanisms; translational repression or mRNA cleavage (Figure 1.3). The mechanism of action is specified by the identity of the target gene. Upon incorporation into the RISC, the mature miRNA will direct mRNA cleavage if its 5' end (seed sequence) possesses sufficient complementarity to the 3' untranslated region (UTR) of the mRNA target (seed match sequence). The profound importance of this complementarity has been underscored by the observation that residues contained within the complementary sites of some of the first validated targets of inverterbrate miRNAs, are perfectly conserved in the orthologous messages of other species (Stark *et al.,* 2003). The specific site where cleavage occurs has been shown to reside between the nucleotides which pair to residues 10 and 11 of the miRNA (Elbashir *et al.,* 2001). Studies have shown that this cleavage site does not shift should the miRNA be imperfectly paired to the target mRNA at the 5' terminus, indicating that the cleavage site is specified by the miRNA residues, rather than the miRNA:target base pair interactions. Following mRNA cleavage, the intact miRNA can go on to drive the destruction of additional target messages.

In the instance that the miRNA does not possess sufficient complementarity to its target, it will direct translational repression. While it has not yet been fully established, many studies have indicated a post-initiation inhibition of mRNA translation model for this form of miRNA-directed regulation (Gu *et al.,* 2009; Petersen *et al.,* 2006; Nottrott *et al.* 2006).

1.7.2 miRNAs and Cancer

In 2002, Calin *et al.* provided the first evidence linking aberrant miRNA expression to tumorigenesis when they demonstrated the frequent downregulation or deletion of a miRNA cluster in chronic lymphocytic leukaemia (Calin *et al.,* 2002). The differential expression of miRNAs was subsequently demonstrated across a panel of diverse human tumour types and it soon became clear that miRNAs were capable of driving malignant progression (Lu *et al.,* 2005). miRNAs have since been shown to be deregulated by a number of structural genetic alterations associated with the development of cancer, such as amplifications, deletions, and chromosomal breakpoints (Michael *et al.,* 2003). Furthermore, miRNA expression can be completely abrogated by promoter DNA hypermethylation and loss of histone acetylation (Cummins *et al.,* 2006). These findings highlight the complicated relationship that exists between miRNA dysregulation and the development of human cancer.

1.7.2.1 Aberrant miRNA Expression in Cancer

While the increased expression of a number of miRNAs has been identified in multiple tumour types, the global reduction of miRNA expression levels is a much more common

occurrence in malignant cells. It remains to be established whether this phenomenon contributes to tumour progression or occurs as a result of the malignant phenotype; however two putative mechanisms for the observed downregulation in miRNA expression patterns have been explored.

Pathologic activation of Myc expression has been demonstrated as a major oncogenic event in human malignancy (Nesbit *et al.,* 1999). Indeed, the Myc transcription factor is widely known to upregulate the pro-tumorigenic miR-17-92 cluster, however studies have revealed that the predominant consequence of Myc activation in human cancer is the global downregulation of miRNA expression (Chang *et al.,* 2008). There is much evidence to support the conclusion that widespread miRNA repression in cancer is mediated by Myc activation. Many of the miRNAs repressed as a result of Myc activation are known to reside in regions frequently deleted in cancer, suggesting that these miRNAs function as tumour-suppressors. For example, members of the *let-7* family of miRNAs which target the *RAS* oncogene are often downregulated in lung cancer (Johnson *et al.*, 2005). These findings highlight the significance of the ubiquitous dysregulation of miRNA expression which, has been observed across multiple cancer types. It would appear that global miRNA repression is not solely an indirect consequence of the malignant state, but rather oncogenic events such as Myc activation reprogram miRNA expression patterns which, further encourages tumorigenesis.

An alternative hypothesis for the marked reduction in miRNA abundance, implicates the constitutive disruption of integral enzymes in the miRNA biogenesis pathway (Lujambio *et al.,* 2012). *In vivo* studies have demonstrated that the deletion of a *Dicer1* allele in lung epithelia promotes *Kras*-driven adenocarcinomas, whereas complete knockdown of *Dicer1* causes lethality due to its critical role in miRNA biogenesis. These findings are also supported within a clinical setting as diminished levels of Dicer and Drosha have been strongly associated with a poor outcome in patients with ovarian cancer (Merritt *et al.,* 2008). Other factors involved in miRNA biogenesis may also be associated with miRNA repression. A study by Melo *et al.* (2009) has identified a number of truncating mutations in *TARBP2*, in both sporadic and hereditary carcinomas. This gene encodes an important component of a *Dicer1*-containing complex and these mutations result in a reduction in TRBP protein levels which, concomitantly disrupts miRNA processing. Overall the profound alterations in miRNA expression levels, which are observed in human cancer, are likely owing to a combination of underlying mechanisms.

1.7.3 miRNAs as Biomarkers

As already stated, miRNAs represent promising biomarkers for diagnosis and prognostication in human cancer and a number of characteristics inherent in these evolutionarily conserved, non-coding RNAs make them particularly suitable for detection in clinical specimens. A comprehensive study by Xi *et al.* (2007) has examined the various potential effects of formalin fixation on the quality and the expression profiles of miRNAs. As the duration of formalin fixation can vary widely among institutions, the putative effects of the formalin fixation process on miRNA quality in mouse liver tissue were examined. Interestingly, the length of fixation time (between 1 and 5 days) was found to have no effect on miRNA stability. The degree of correlation in miRNA expression profiles between FFPE (formalin-fixed paraffin-embedded) specimens and fresh frozen tissue samples was also examined and a favourable correlation was found between the two. Furthermore, it was demonstrated that miRNAs from archival FFPE specimens remain stable over a ten year period indicating that miRNA profiling of archival clinical FFPE specimens is entirely feasible. Infact, miRNA profiling is believed to be more accurate than mRNA profiling, in that miRNA expression is unique to the cellular origin of tumours and can often be used to classify poorly differentiated tumours when standard histological techniques fail (Lu *et al.,* 2005). Metastatic cancer of unknown primary origin is believed to account for 3-5% of newly diagnosed cancer cases and treatment of this form of disease represents a major clinical dilemma (Pimiento *et al.,* 2007). However, miRNAs have recently emerged as promising candidates for the identification of cancer tissue of origin in such cases.

miRNA expression profiling as a diagnostic tool is not restricted to solid tumours, as the discovery of tumour-derived circulating miRNAs in serum has proved. miRNAs have been shown to possess exceptional stability in clinical plasma and serum samples, providing an exciting avenue for the development of miRNAs as novel blood-based biomarkers. For example, miR141 has been shown to be more highly expressed in the serum of patients with metastatic prostate cancer than in healthy age-matched control individuals (Mitchell *et al.,* 2008). While this area of biomarker discovery is in its infancy, the successful development of this technology could provide a clinically-feasible, minimally-invasive diagnostic test for a range of cancer types, including prostate cancer.

1.7.4 miRNA Expression Signatures in Prostate Cancer

The first prostate cancer-specific miRNA signature was described in a study carried out by Porkka *et al.,* (2007) in which the expression of 319 miRNAs was examined in a variety of prostate cancer cell lines and clinical prostate samples. The differential expression of 51 miRNAs was found to accurately separate benign prostatic hyperplasia from carcinoma

samples. Moreover, these unique miRNA expression profiles could further classify the carcinoma samples according to their androgen dependence. A subsequent study by Ozen *et al.,* (2008) examined the expression of 480 miRNAs in a well characterised cohort of prostate cancers comprising 10 benign peripheral zone samples and 16 radical prostatectomy specimens. The results of this analysis demonstrated a widespread statistically significant downregulation of miRNA expression in organ-confined prostate cancer. The loss of a number of biologically relevant miRNAs (miR-125b, miR-145 and let-7c) was subsequently validated in a larger cohort using quantitative RT-PCR. Furthermore, in an attempt to examine the effects of miRNA expression on mRNA levels in prostate cancer, the expression of putative gene targets of miR-125b was analysed. 26 (of a possible 286) targets of miR-125b were found to be upregulated in prostate cancer tissue, the most notable being several genes involved in the control of translation, including EIF4EBP1, RPL29 and PAPB. The findings of this study are consistent with that of Porkka *et al.* (2007) indicating a global downregulation of miRNAs in prostate cancer, however these studies do not shed light on the possibility of further miRNA dysregulation as cancer progresses to metastatic disease, nor do they explore the putative mechanism underlying this observed widespread dysregulation of miRNAs.

In 2009, Tong *et al.,* performed the first paired analysis of microdissected malignant and non-involved areas of 40 radical prostatectomy specimens. The expression of 114 miRNAs was examined, five of which, (miR- 23b, -100, -145, -221, and -222) were found to be significantly downregulated in tumour tissue. The ectopic expression of these miRNAs was shown to disturb the growth of LNCaP prostate cancer cells, indicating a growth regulatory function for these miRNAs. Furthermore, biochemical recurrence within 2 years of surgery in a subset of patients was found to correlate with a distinct expression profile of 16 miRNAs, which is highly suggestive of a dynamic miRNA expression profile as the malignant state progresses. These results provided the first evidence of the prognostic value of miRNAs in prostate cancer.

A further study by Spahn *et al.,* (2010) implicated miR-221 as a novel prognostic indicator in high-risk prostate cancer. This study employed microarray analysis to examine the global expression of miRNAs in benign prostatic hyperplasia (BPH) tissue, primary prostate cancer in a subset of high risk cases and corresponding metastatic tissue. The aberrant expression of a number of miRNAs was identified, including the profound downregulation of miR-221 in metastatic prostate cancer. The observed downregulation of miR-221 was found to be associated with malignant progression and clinical recurrence in a subset of high-risk patients. Downregulation of miR-221 has previously been associated with the growth of erythroleukemic cells mediated by the loss of the proto-oncogene *c-kit* (Felli *et al.,* 2005).

This relationship was further validated by Spahn *et al.,* (2010) as this study also demonstrated that the downregulation of miR-221 correlates with the upregulation of *c-kit* mRNA. It is prudent to note, that prostate epithelial cells only weakly express *c-kit*, however in an experimental model of prostate cancer bone metastasis; bone tumours formed by exclusively c-kit-negative PC-3 cells were found to strongly express *c-kit* (Wiesner *et al.,* 2008). These results combined with the observations made by Spahn *et al.,* (2010) suggest a mechanism of increased *c-kit* expression in prostate cancer progression, however additional investigation is required to confirm this hypothesis. This study by Spahn *et al.,* (2010) was the first to identify a single miRNA candidate of significant prognostic value, however further studies have yet to support the utility of miR-221 as a potential therapeutic target in prostate cancer.

More recently, the diagnostic and prognostic significance of a panel of miRNAs was examined in a study by Schaefer *et al.,* (2010). The expression of a panel of differentially-expressed miRNAs was analysed in a cohort of 76 matched tumour and normal prostatectomy specimens. The expression of five miRNAs were found to correlate with Gleason score and tumour stage. In particular, the upregulation of miR-96 was significantly associated with biochemical failure following radical prostatectomy. miR-96 has previously been shown to be upregulated in chronic myeloid leukaemia cells (Agirre *et al.,* 2008). Furthermore, increasing miR-96 expression was found to correlate with a concomitant decrease in time to recurrence following radical prostatectomy.

Various studies have documented the presence of tumour-derived miRNAs in circulation, enhancing their desirability as non-invasive biomarkers for diagnosis. The precise mechanisms whereby miRNAs are secreted into circulation remain incompletely understood. Many posit that they merely seep into circulation due to the natural tissue disruption, which accompanies tumorigenic progression, however increasing evidence is supporting the assertion that miRNAs are actively deposited into circulation within tumour-derived exosomes (Taylor *et al.,* 2008). Exosomes are small membrane vesicles of endocytic origin, whose accumulation in peripheral circulation appears to be confined to cancer (Taylor *et al.,* 1979). These vesicular bodies can be released by multiple proliferating cell types; however it is believed they are more commonly released by tumour cells as evident by their elevated levels in the plasma of cancer patients. As previously mentioned, a study by Mitchell *et al.,* (2008) analysed the serum from 25 patients with metastatic prostate cancer and 25 healthy age-matched controls. The differential expression of 5 miRNAs was observed between the two groups, (miR-100b, miR-125b, miR-141, miR-143, and miR-296). In particular, the expression of miR-141 was found to be enriched in the serum of patients with metastatic disease when compared to the healthy individuals. Moreover, the levels of miR-141 in serum

were found to be capable of detecting prostate cancer with 100% specificity and 60% sensitivity. These findings were repeated in a study by Brase *et al.,* (2011), which reported a higher abundance of miR-141 in the serum of patients with advanced disease when compared to those with low-grade tumours.

A study by Zhang *et al.,* (2011) sought to assess the role of circulating miR-21 in the progression of prostate cancer. Serum samples drawn from 56 patients were examined. This sample set comprised; 20 patients with organ-confined prostate cancer, 20 patients with androgen-dependent disease, 10 patients with hormone-refractory prostate cancer (HRPC) and 6 patients with benign prostatic hyperplasia (BPH). The HRPC patients were treated with docetaxel chemotherapy. miR-21 expression was found to be upregulated in the serum of patients with hormone-refractory disease. Furthermore, those patients who were resistant to docetaxel-based treatment exhibited the highest levels of circulating miR-21. While this study was based upon a small sample size, the results indicate the potential of miR-21 as a serum-based predictor for the efficacy of docetaxel treatment regimes.

The potential of miR-21 as a diagnostic biomarker was further recognised in a study by Yaman *et al.,* (2011). This study examined plasma from a cohort of 51 patients comprising two groups of localised and metastatic disease. The expression of miR-21 was found to be sufficient to discriminate between the patient group and the healthy controls. These findings correlate with previous studies, which report the marked upregulation of miR-21 in prostate tumours. However, the expression levels of miR-141 were found to contradict previous studies, which have reported an abundance of miR-141 in the serum of prostate cancer patients compared to healthy individuals. This discrepancy may be due to the use of plasma in this study, as serum is known to contain much higher quantities of circulating nucleic acids (Umetani *et al.,* 2006). However, in keeping with previous reports, higher levels of miR-141 were identified in patients with metastasis when compared to those with localised prostate cancer.

While the study of circulating miRNA expression has yielded varied and highly interesting results, they must be viewed with caution. At present there is a lack of consistency regarding the optimum circulating medium from which to quantify miRNAs, extraction techniques and analytical methods. Many studies to date have focussed on the extraction of RNA from exosomes and serum or plasma; however a recent study by Heneghan *et al.,* (2010) has optimised an RNA extraction technique from whole-blood samples, which yields a higher amount of miRNAs compared to matched serum and plasma samples. There remains no consensus regarding this issue, which likely accounts for the lack of translation of potential circulating miRNA biomarkers into clinical utility.

A recent study by Srivastava *et al.,* (2013) has highlighted the potential diagnostic significance of urinary miRNAs. This study employed laser capture microdissection to isolate specific cell populations from a cohort of formalin-fixed paraffin-embedded prostate tumour specimens. Global miRNA expression profiling was performed in an effort to identify miRNAs associated with the development of prostate cancer. The miRNA candidates were subsequently validated in an independent set of urine samples from prostate cancer patients. A number of miRNAs (miR-205, miR-221, miR-214, and miR-99b) were identified as significantly downregulated in tumour tissue when compared to surrounding normal tissue, the most significant of which were miR-205 and miR-214. The loss of miR-205 in prostate cancer has previously been associated with a poor prognosis and is also believed to be involved in epithelial-mesenchymal transition (EMT) (Hagman *et al.,* 2013). Furthermore, aberrant miR-205 expression has been identified as a prognostic marker in head and neck squamous cell carcinoma and aggressive bladder tumours, which further confirms its pathological role in the development of cancer. This study was the first to report the aberrant expression of miR-214 in prostate cancer; however its loss has previously been reported in a number of gynaecological malignancies (Wang *et al.,* 2013; Vaksman *et al.,* 2011). Analysis of these miRNAs in a set of urine samples revealed a concordant expression profile; miR-205 and miR-214 were found to be significantly downregulated in the urine of prostate cancer patients. Furthermore, the expression of these miRNAs could discriminate patients from healthy control individuals with a high degree of specificity.

Much like miRNAs in circulating serum and plasma, urine miRNAs display a robustness, which advocates their use as tumour biomarkers; however this area of study is relatively nascent. At present, there is no sufficiently comprehensive study which describes the potential of urine-derived miRNAs as novel diagnostic and prognostic biomarkers. However, it is hoped that further investigations into the area of tissue-based and circulating miRNAs will fuel the assertion that specific miRNA expression profiles can be harnessed as molecular markers and translated into non-invasive clinical screening tools.

1.8 Cancer Stem Cells

Emerging evidence supports the concept that a biologically distinct population of cells, termed cancer stem cells are integral to the initiation and perpetuation of several forms of human cancer. As the existence of cancer stem cells is being increasingly substantiated by experimental evidence, it is becoming crucially important to deepen our understanding of the molecular mechanisms which regulate key features of cancer stem cells. Thus, the targeting and eradication of cancer stem cells has become an area of intense interest in recent years.

Normal stem cells possess three distinctive properties; a profound proliferative capacity, the capability to develop along multiple lineage pathways and most importantly the ability to self-renew (Jordan *et al.,* 2006). Asymmetric division inherent in stem cells gives rise to two daughter cells; a new stem cell and a progenitor cell which can differentiate and proliferate but does not possess the ability to self-renew. Many studies, which have identified rare 'tumour-initiating' cells in cancers of the hematopoietic system, breast and brain, have demonstrated the relevance of stem cell characteristics to the pathology of human cancer (Al-Hajj *et al.,* 2003; Singh *et al.,* 2003). Indeed, 'tumour-initiating' cells also have the propensity to self-renew and possess the proliferative power to drive malignant progression, thus cells of this type have been termed "cancer stem cells". Disruption of the critical self-renewal potential intrinsic in cancer stem cells combined with their powerful proliferative capacity is largely believed to account for a malignant phenotype.

1.8.1 The Origin of Cancer Stem Cells

The cancer stem cell hypothesis postulates that much like the normal stem cell compartment which maintains the tissue of an organism, a rare subset of cells is responsible for maintaining tumour growth and driving metastasis (Lobo *et al.,* 2007). Two potential theories have been put forth to explain the origin of cancer stem cells. It was initially believed that cancer stem cells arose solely from the malignant transformation of normal stem cells due to seminal work by Lapidot *et al.,* (1994). They identified a putative leukaemia initiating-cell based on cell surface marker expression, ($CD34^+$ $CD38^-$), which was capable of recapitulating disease features in NOD/SCID mice. As the leukaemia-initiating population resembled the phenotype of normal hematopoietic stem cells, they postulated that leukemic stem cells arise from the transformation of normal blood stem cells. However a study by Tavil *et al.,* (2006) has demonstrated that the increased expression of surface markers CD34/CD117, which is characteristic of leukemic stem cells, is infact more similar to the phenotype of the progenitor cell. Further evidence from mouse leukemia models would appear to substantiate a progenitor origin for cancer stem cells. A study by Krivstov *et al.,* (2006) has demonstrated that a committed progenitor cell can be transformed to a leukemic stem cell through the introduction of the MLL-AF9 fusion gene (a leukemogenic fusion protein which promotes self-renewal). Analysis of leukemia stem cells in human chronic myelogenous leukemia has provided further evidence to support the ability of progenitor cells to give rise to pathologic stem-like cells. Jamieson *et al.,* (2004) have demonstrated that in chronic-phase CML only cells resembling normal blood stem cells are capable of yielding self-renewing colonies. However, the opposite was observed in blast-crisis CML; malignant cells possessing a phenotype similar to that of the differentiated progenitor cell could acquire the capacity to self-renew. These results indicate that both modalities of stem-

like cell generation may occur in human cancer. Overall, these findings are highly suggestive that in order to become a cancer stem cell, a progenitor cell must harbour a range of mutations that result in its de-differentiation and subsequent acquisition of stem cell characteristics, such as the ability to self-renew. Thus, it is likely that multiple diverse pathways can generate cancer stem cells.

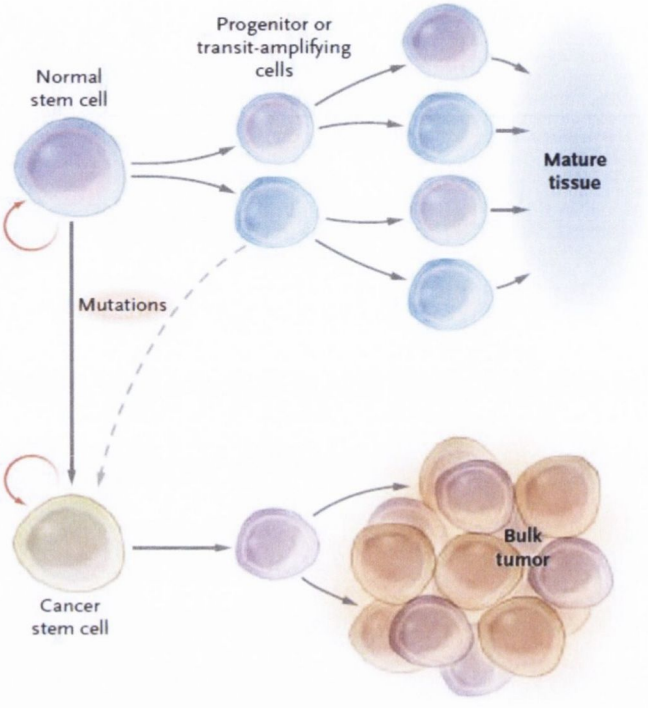

Figure 1.4 *Normal and Cancer Stem Cells.*

Both normal and cancer stem cells possess the critical ability to self-renew, indicated by red arrows. Cancer stem cells may arise from mutations within normal stem cells or progenitor cells. Adapted from Jordan *et al.,* (2006).

1.8.2 The Origin of Prostate Stem Cells

The prostate gland is composed of three distinct epithelial cell populations; basal cells, neuroendocrine cells and luminal secretory cells (Abate-Shen *et al.,* 2000). Luminal cells are polarised columnar cells characterised by high expression of the androgen receptor (AR), prostate specific antigen (PSA), prostatic acid phosphatse (PAP) and low molecular weight keratins (K8 and K18) (Sar *et al.,* 1990). This population of cells is responsible for secreting prostatic proteins into the glandular lumina and is the most predominant component of both normal and malignant prostate. Basal cells, which are relatively undifferentiated line the basement membrane of each prostatic duct and primarily express high molecular weight

keratins (K5 and K15) and p63. Basal cells express low or undetectable levels of the androgen receptor and consequently do not depend on androgen for their growth (Kyprianou *et al.,* 1988). Epithelial cell growth is sustained by neuroendocrine peptides released by neuroendocrine cells, which also line the basement membrane (Bonkhoff *et al.*, 1995).

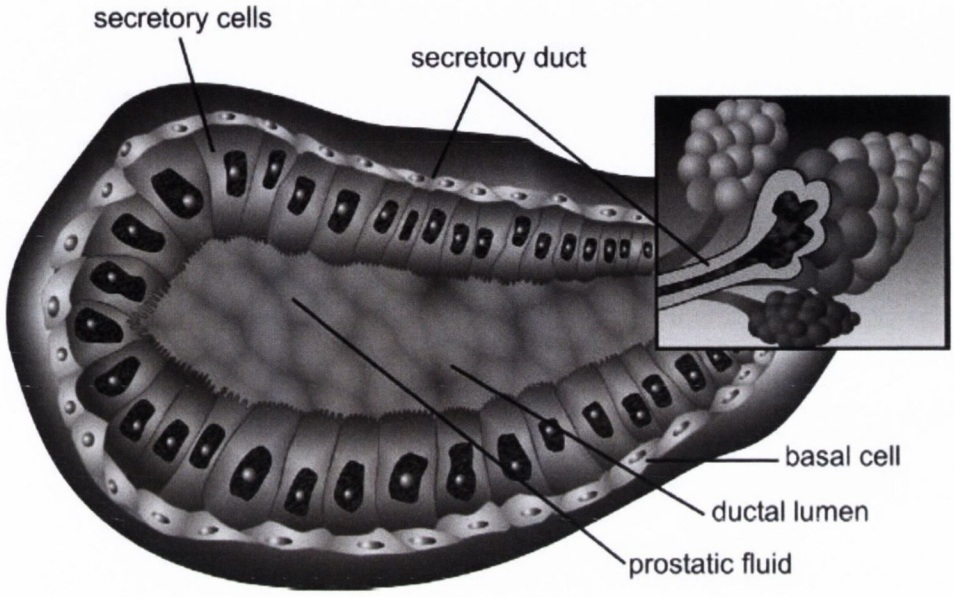

Figure 1.5 *Prostate gland architecture.*

A cross section of the ductal region of the prostate with labels indicating the predominant cellular phenotypes. Adapted from Collins *et al.,* (2006).

Isaacs and Coffey (Isaacs & Coffey, 1989) were the first to propose the existence of prostate stem cells. As previously mentioned, the prostate is an androgen-dependent organ that undergoes involution upon androgen ablation. It has been demonstrated that castration of the male rat results in the loss of 90% of the total epithelial cells within the prostate (Kyprianou *et al.,* 1988). However, upon androgen restoration the prostate can completely regenerate. This regression-regeneration process can be repeated for up to 30 cycles, which has led to the hypothesis that the prostate contains a population of long-lived, androgen insensitive epithelial stem cells, whose proliferative potential is capable of solely orchestrating repopulation. Tsujimura *et al.,* (2002) have proposed that prostate stem cells may be located in the proximal region of the mouse prostate based upon the observation that this area enriches for cells possessing a stem phenotype. Although it remains to be conclusively determined whether the prostate stem cell population possesses a basal or a luminal phenotype, there is significant evidence to support both arguments.

It has been demonstrated that androgen deprivation causes the vast majority of luminal cells (90%) to undergo apoptosis indicating that normal prostate stem cells may have a basal-cell identity (English *et al.,* 1987). Furthermore, both human and rodent basal cells possess a much stronger proliferative capacity than luminal cells (Bonkhoff & Remberger, 1996). The identification of cells representing a phenotypic intermediate between basal and luminal cells, which co express both basal-specific and luminal specific cytokeratins has provided further evidence supporting the hypothesis that basal cells are the progenitors of luminal secretory cells and that this intermediate cellular phenotype represents the transit-amplifying population (Peehl *et al.,* 1994). PSCA (prostate cancer stem cell antigen), a putative marker of these intermediate cells has been shown to be upregulated in prostate tumours indicating that prostate adenocarcinomas may infact arise from these intermediate transit-amplifying cells (Tran *et al.,* 2006; Reiter *et al.,* 1998).

Conversely, there is increasing evidence to support a luminal phenotype for normal prostate stem cells. The bulk population of malignant cells in human prostate adenocarcinomas express luminal cell-specific markers whereas cells expressing basal-cell markers are rarely observed, which is consistent with the notion that prostate cancer arises from a luminal cell displaying stem properties (Okada *et al.,* 1992). Brd-U pulse-chase experiments conducted by Tsujimura *et al.,* (2002) have provided evidence to substantiate the existence of stem-like cells within the luminal cell population. Another important study by Kurita *et al.,* (2004) has demonstrated that tissue from $p63^{+/+}$ and $p63^{-/-}$ embryos can reliably generate prostate tissue when grafted into adult male nude mice. Tissue from p63-deficient grafts was found to contain both neuroendocrine cells and luminal cells but lacked basal cells. Furthermore, castration was found to cause a severe regression of the $p63^{-/-}$ prostate; however this tissue did regenerate in response to androgen stimulation. These results would appear to indicate that basal cells are infact not required for prostatic regeneration. More recent work by Wang *et al.,* (2009) has also argued against a basal phenotype for prostate stem cells. This study has identified castration resistant Nkx3.1-expressing cells (CARNS) in androgen-deprived prostate epithelium, which comprise 0.7% of the epithelial cells within the prostate. This cellular population has demonstrated an *in vivo* self-renewal capacity and has been implicated in prostatic regeneration. Most notably however, CARNS display a luminal cell phenotype.

1.8.3 The Origin of Prostate Cancer Stem Cells

Several characteristics inherent in human prostate cancer indicate a normal prostate stem cell origin for this disease. As already mentioned, while androgen ablative therapy generally causes an initial widespread regression of tumour cell growth, the development of hormone-

refractory disease is inevitable. This has prompted speculation that prostate tumours may contain a small population of androgen-independent cells that are not eradicated by initial treatment strategies (Litvinov *et al.,* 2003). This reservoir of cells could potentiate regrowth of the tumour in the absence of androgen. As normal prostate stem cells are androgen insensitive they provide an attractive candidate as the source of prostate cancer stem cells (Lawson *et al.,* 2007). It remains to be fully elucidated whether differentiated cells within a prostate tumour are capable of generating new tumour cell progeny or infact there exists a subpopulation of cancer stem cells within the bulk tumour which possesses the propensity for transformation. The hierarchical model, which predicts that this tumour-initiating subset of cells is distinct from the main population of the solid tumour, would in part explain the functional heterogeneity observed in prostate cancer. The results of numerous efforts to isolate and characterise prostate cancer stem cells would appear to support the latter hypothesis, that there exists a stem cell niche within the tumour, which acts as the source of all malignant cells and orchestrates metastasis. Furthermore, contrary to the popular belief that prostate cancer arises from luminal cells as the disease is characterised by the absence of basal cells, there is growing evidence to support the ability of basal cells to initiate tumorigenesis.

1.8.4 Identification and Isolation of Prostate Cancer Stem Cells

Patrawala *et al.,* (2006) were the first to report the identification of prostate cancer stem-like cells. This study focussed on the somewhat controversial involvement of the adhesion and signalling molecule CD44 in prostate cancer development and progression. Many studies have examined the expression and putative function of CD44 in prostate carcinogenesis yielding conflicting and largely equivocal data. For example, an early study by Kallakury *et al.,* (1996) reported the complete loss of CD44 expression in prostate cancer tumours, whereas a study by Nagabhusan *et al.,* (1996) has reported a marked overexpression of CD44 in primary prostate neoplasms. Furthermore, a study by Gao *et al.,* (1997) has shown that the ectopic expression of CD44 causes tumour-suppressive effects. However, a number of other studies have disputed these findings and demonstrate the prominent role of CD44 in proliferation, invasion and metastasis (Paradis *et al.*, 1998; Lokeshwar *et al.* 1995). A study by Patrawala *et al.,* (2006) employed FACS to isolate homogeneous $CD44^+$ and $CD44^-$ cell populations from prostate cancer cell lines and a number of xenograft tumours. The highly purified $CD44^+$ prostate cancer cells were found to exhibit a higher proliferative and clonogenic capacity when compared to their negative counterparts. Furthermore, orthotopic implantation experiments were performed to assess the *in vivo* tumour-forming potential of homogeneous $CD44^+$ cells and these cells were found to be vastly more tumorigenic than CD44-negative cells. Moreover, $CD44^+$ cells were capable of initiating disseminated

metastasis upon implantation, while no metastatic spread was observed in the $CD44^-$ xenografts. $CD44^+$ cells were also shown to be AR^- and to overexpress at the mRNA level a number of critical stem-associated genes including OCT3/4, Bmi and β-catenin, indicating that prostate cancer may arise from a basal cell origin.

A study by Collins *et al.,* (2001) reported the isolation of prostate stem cells from normal prostate epithelia based on the expression of putative stem markers integrin α2β1 and CD133 (Richardson *et al.,*2004). The same technique was subsequently applied to prostate tumours of differing Gleason grade and metastatic status in an attempt to isolate potential cancer stem cells (Collins *et al.,* 2005). Collins *et al.,* (2005) reported the identification and characterisation of cells possessing an antigenic profile analogous to that of putative normal epithelial stem cells. These cells were also found to possess the same stem-associated properties, most notably self-renewal. This population of cells, of the phenotype $CD44^+$/integrin $\alpha2\beta1^{high}/CD133^+$ were shown to have a high proliferative and clonogenic capacity *in vitro*. In order to ensure that these cells originated from tumour and not from contaminating normal stem cells, *in vitro* invasion assays were performed. The $CD133^+$ population of cells were found to be significantly more invasive than the metastatic positive controls. Furthermore, this cellular population was shown to be negative for androgen receptor expression. Thus, Collins *et al.,* (2005) proposed that prostate tumours arise from $CD133^+$ basal cells as this population retains the ability to self-renew and give rise to phenotypically diverse populations of differentiated cells. This work is highly suggestive that the observed heterogeneity and androgen-sensitivity, which characterises prostate cancer is a result of differentiation from an androgen-insensitive stem cell population. A more recent study by Goldstein *et al.,* (2010) has again challenged the belief that luminal cells are the putative cells of origin for human prostate cancer. Two distinct basal ($CD49f^{lo}Trop2^{hi}$) and luminal ($CD49f^{hi}Trop2^{hi}$) cell populations from primary benign human prostate tissue were combined with mouse urogenital sinus mesenchyme (UGM) for transplantation into the subcutaneous space of NOD-SCID-IL-$2R\gamma^{null}$ mice. Interestingly, only basal cells were capable of regenerating benign prostatic tissue. Furthermore, adenocarcinoma was found to develop upon introduction of oncogenic alterations to the basal cells, namely the activation of Akt, ERG and AR.

A study by Gu *et al.,* (2007) has also addressed the lineage status of prostate cancer stem cells. This study demonstrated that clonally-derived human telomerase reverse transcriptase-immortalised (hTERT) primary prostate cancer cell lines were capable of recapitulating histopathological features of the original prostate tumours from which they were derived, in mice. They also displayed multilineage differentiation *in vivo* to generate basal, luminal and neuroendocrine cells of the normal prostate. Furthermore, these cells

were found to highly express embryonic pluripotent stem cell markers OCT4, NANOG and SOX2 and as in previous studies, they were found to be negative for AR and p63.

Xin *et al.,* (2005) have reported the significance of Sca-1 (stem cell antigen 1) a glycosylphosphatidylinositol-linked cell surface protein, in the isolation of prostate cancer cells with stem cell-associated properties. This protein has previously been shown to enrich for somatic stem cells in a number of other tissue types (Spangrude *et al.,* 1988; Welm *et al.,* 2002). Xin *et al.,* (2005) have demonstrated that Sca-1 enriches for murine prostate cells capable of regenerating prostatic structures comprised of basal and luminal cell lineages in tissue recombination assays. Interestingly, Sca-1^+ cells were localised to the proximal region of prostatic ducts where low-cycling putative prostate stem cells have previously been identified (Tsujimura *et al.,* 2002). The Sca-1^+ prostate-regenerating cells were also shown to be androgen-insensitive, which correlates with previous studies. These findings indicate that the Sca-1 surface antigen may be utilised to identify cells displaying stem-like properties, including multilineage differentiation and self renewal.

While a number of protein expression signatures have been successfully utilised to identify populations of prostate cells enriched for a stem phenotype, true prostate cancer stem cells have yet to be characterised in great detail. As the hierarchy of human prostate epithelial cells is further unravelled, more tumour-initiating cell populations may be identified. While prostate cancer has long been histologically classified by its lack of basal cells, it is prudent to note that this characterisation does not necessarily reflect the cellular origin of the disease. There is increasing evidence to support the association between androgen independent basal cells and residual disease following hormone ablative treatment. This would indicate that pathways involved in normal basal cell function may play a role in potentiating tumour survival and metastasis following androgen-deprivation treatment. It is hoped that the continued study of prostate cancer stem cells may reach a consensus on the prostate cancer cell of origin and delineate the crucial underlying mechanisms by which these cells operate.

1.8.5 Prostate Cancer Stem Cells and Treatment

1.8.5.1 The Role of Prostate Cancer Stem Cells in Diagnosis and Prognosis

The cancer stem cell hypothesis has profound clinical relevance to risk assessment and prediction in human cancer. As discussed in 1.6.2, critical diagnostic and predictive information can be garnered from the molecular expression profile of prostate tumours. Indeed, miRNA expression profiling can even reflect the cellular origin of disease (1.7.3). Thus, it has been postulated that the identification of ncRNA and gene signatures

characteristic of cancer stem cells may yield important information regarding disease behaviour and patient outcome. A study by Glinsky *et al.,* (2005) has demonstrated the consistent elevation of stem cell-associated gene *BMI-1* in prostate carcinomas. This gene has previously been implicated in determining the proliferative potential of both normal and leukemic stem cells (Lessard *et al.,* 2003). Glinsky *et al.,* (2005) identified a stem cell-like *BMI-1*-associated 11-gene signature in primary prostate tumours, which is predictive of therapy failure. Furthermore, this signature was found to be a strong predictor of short interval to disseminated metastasis and poor prognosis following treatment. These results provide convincing evidence for the use of stem-cell associated molecular patterns in the characterisation of disease subtypes, however in order to yield a more valuable therapeutic target it will most likely be necessary to profile purified cancer stem cell populations.

The proportion of cancer stem cells present within the bulk tumour has previously been shown to predict disease behaviour in a number of other malignancies, including glioblastoma (Singh *et al.,* 2003). For example, in medulloblastomas and astrocytomas the cancer stem cell population has been shown to range from 3.5% to 46.3% of the bulk tumour. Interestingly, there is much fewer cancer stem cells present in low-grade astrocytomas when compared to high-grade medulloblastomas. This has led to the hypothesis that a large cancer stem cell population predisposes a more aggressive, invasive disease and ultimately a poor prognosis. However, initial experimentation using prostate cancer specimens has failed to establish a link between the cancer stem cell load and Gleason grade.

In recent years, there has been growing interest in the area of circulating tumour cells (CTCs). It is hoped that the isolation and characterisation of these cells from patients with varying disease severity will inform treatment selection and elucidate mechanisms of resistance to standard treatment modalities. Indeed, a number of previously published studies have demonstrated that the enumeration of CTCs in breast, colon and prostate cancer has a prognostic benefit (Hardingham *et al.,* 2000; Moreno *et al.,* 2005, Cristofanilli *et al.,* 2004; Budd *et al.,* 2006; Garcia *et al.,* 2007). It has been postulated based on the tumour-initiating potential of cancer stem cells that circulating tumour cells may provide an attractive source of cancer stem cells. Unfortunately, Danila *et al.,* (2007) have demonstrated that CTCs isolated from castration-resistant prostate cancer patients are likely to be terminally differentiated and highly express cytokeratins (8, 18, and 19) and PSA (Smirnov *et al.,* 2005). While the observation that circulating tumour cells express *AMACR* and contain genetic abnormalities associated with prostate cancer has substantiated their primary tumour origin, the proportion of stem cells present is likely to be very low (Schwarzenbach *et al.,* 2007). The scarcity of cancer stem cells not only in solid tumours but

also in circulation is likely to hamper the manipulation of this cellular population for prognostication.

As previously alluded to, disease progression and conventional treatment failure are attributed to the existence of cancer stem cells. The hypothesis is that much like normal stem cells which are known to be resistant to the induction of apoptosis by cytotoxic drugs, this rare population of cells can form a drug-resistant reservoir, which if not eradicated during initial treatment, can cause disease recurrence (Figure 1.6). For example, Guzman *et al.*, (2002) have demonstrated that leukemic stem cells are inherently more resistant to chemotherapeutic agents than differentiated myeloblastic cells. It is hoped that the *in vitro* study of cancer stem cells and the subsequent isolation of cancer stem cells from primary and metastatic tumours may facilitate the delineation of the biologic mechanisms by which this rare subpopulation confers drug resistance. A full understanding of how cancer stem cells function may allow the development of novel stem-cell directed therapies.

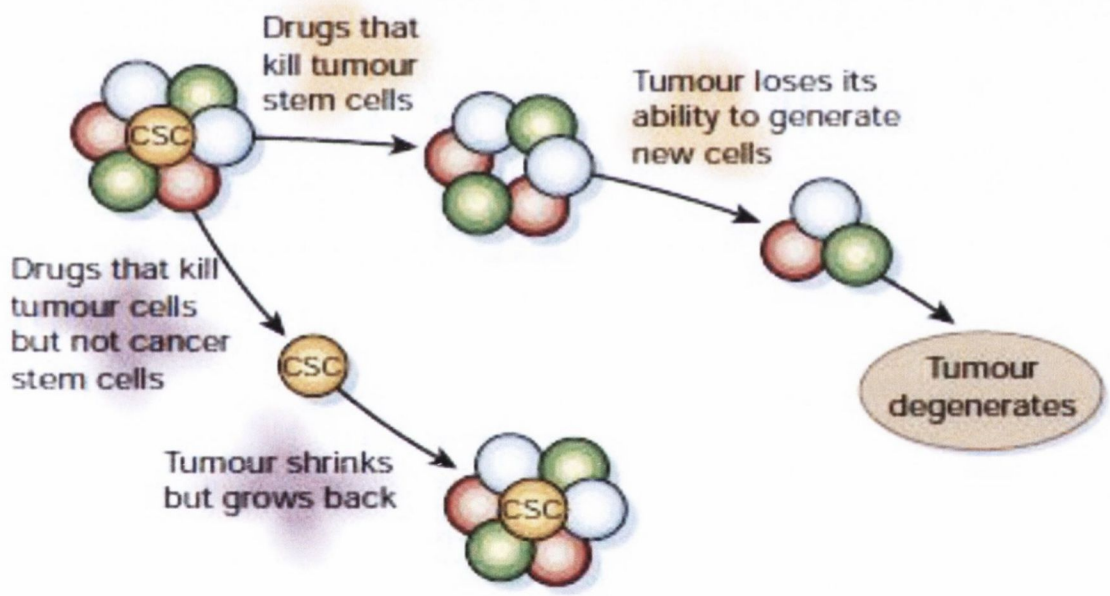

Figure 1.6 *Cancer stem cells and conventional therapy.*

Standard therapeutics may cause initial tumour regression by eradicating cells of a limited proliferative index. However, failure to eliminate the cancer stem cell population will allow this cellular niche to regenerate the tumour. Conversely, stem cell-targeted therapies may ultimately achieve a cure by abrogating malignant expansion. Adapted from Reya *et al.*, (2001).

1.8.5.2 Targeting Prostate Cancer Stem Cells

The cancer stem cell model indicates that the eradication of cancer may rely on the elimination of the cancer stem cell population. At present, it remains to be established what morbidities, if any, are associated with targeting stemness. In addition, a major challenge to devising stem-targeting therapeutics will be the selective removal of the cancer stem cell population while sparing normal somatic stem cells. Many of the pathways which orchestrate self-renewal and multilineage differentiation in normal stem cells are hypothesised to be common to their oncogenic counterparts. A number of preliminary studies have explored the feasibility of targeting the cancer stem cell population. Notch signalling is mediated through the action of the γ-secretase enzyme, and a study by Weijzen *et al.,* (2002) has demonstrated the efficacy of inhibiting this enzyme in breast cancers where Notch1 is overexpressed. The inhibition of Hedgehog by the agent cyclopamine has also been demonstrated to cause regression of prostate xenograft tumours in athymic mice (Karhadkar *et al.,* 2004). The results of this study indicate that tumour-initiating cells require the Hedgehog signalling pathway to function effectively. It is prudent to note that the expression of the polycomb group protein BMI-1 is also dependent upon the Hedgehog pathway. Taken together, this data implicates the Hedgehog pathway in the propagation of malignant prostate cells, and in the maintenance of putative self-renewing cancer stem cells. Further investigation is required in order to validate these results, but preliminary data would suggest that the manipulation of the Hedgehog pathway may provide a basis for therapeutic elimination of the prostate cancer stem cell population.

1.9 Conclusion

Prostate cancer is a naturally heterogeneous malignancy with a protracted clinical course. A post-mortem study by Sakr *et al.,* (1994) has demonstrated that a large proportion of men over 50 harbour prostate adenocarcinoma upon histological examination of the prostate. This would indicate that prostate cancer is relatively indolent in nature; however the American Cancer Society has published statistics which state that of the total number of prostate cancer cases diagnosed in 2013, 12% of these resulted in patient mortality. Indeed, prostate cancer is a leading cause of cancer-related mortality. These contradictory facts underscore the major clinical challenges, which currently hamper the effective management of prostate cancer. At present, it is not possible to adequately stratify patients based on their risk of progression. The inability to confidently delineate at diagnosis clinically indolent from aggressive prostate tumours has led to the majority of patients receiving aggressive treatment and consequently resulted in the widely accepted problem of overtreatment. A study by Holmberg *et al.,* (2002) examining the benefit of radical prostatectomy versus

watchful-waiting found only a 6.6% reduction in mortality following prostatectomy when compared with active surveillance, indicating that the benefit of radical surgery is confined to a subset of as yet undefined prostate cancer patients. While the introduction of active surveillance has attempted to address the burden of this problem, the lack of standardised methods to monitor disease progression means the window of time in which curative treatment is possible, may be missed. Furthermore, as the disease progresses treatment options become limited and patients with advanced, late-stage disease have a poor prognosis. Until recently the ability to address the major clinical challenges in prostate cancer was limited as analytical methods were confined to histology and immunohistochemistry; however a number of critical technological advancements have entirely altered our understanding of the biologic mechanisms of cancer and created new optimism in the search for improved diagnostic and prognostic biomarkers in prostate cancer. Furthermore, we are gaining a deeper understanding of disease characteristics and behaviour through the study of rare tumour-initiating cell populations known as cancer stem cells. The fundamental aim of this project is to marry these two concepts in an attempt to not only molecularly define aggressive prostate cancer but also further understand the mechanisms, which govern the heterogeneity of this malignancy.

1.9.1 Hypothesis & Project Aims

The hypothesis of this project is that there exists a specific molecular signature, which defines clinically aggressive prostate cancer. Furthermore, the existence of a rare prostate cancer stem cell population within bulk tumours is hypothesised to perpetuate tumour growth, confer resistance to treatment, direct metastasis and ultimately predict a poor prognosis.

The fundamental aims of this project were;

- To perform a large-scale expression analysis of a panel of genes and miRNAs using archival formalin fixed paraffin embedded prostatectomy specimens drawn from the Prostate Cancer Research Consortium (PCRC) biobank. These samples have been divided into putatively indolent, significant and aggressive disease based on Epstein's Criteria for clinically insignificant disease. The panel of interest contains genes and miRNAs, which have been identified through previous studies performed within the department and a meta-analysis of relevant literature. It is hoped that the identification of a signature capable of delineating clinically indolent from aggressive disease will go some way to elucidating the mechanisms, which govern aggressive prostate cancer.

- To examine the expression of a number of downstream targets of the miRNA panel of interest in a tissue microarray composed of a training set of patient samples.

- To optimise a robust method of prostate cancer stem cell enrichment from immortalised prostate cancer cell lines, in order to generate an *in vitro* prostate cancer stem cell model.

- To analyse the expression of a number of critical prostate cancer stem cell-associated markers, in addition to known embryonic stem cell pluripotency markers in these putative cancer stem cells.

- To examine the *in vivo* tumour-initiating capacity of these putative cancer stem cells through murine transplantation assays.

- To perform next-generation sequencing of the coding and non-coding RNA repertoires of prostate cancer stem cells, in order to unravel the potential biologic mechanisms, which underpin critical stem cell-associated features such as self-renewal and multilineage differentiation.

Materials and Methods

Chapter 2

Chapter 2. Materials and Methods

2.1 Cell Culture

2.1.1 Cell Culture Conditions

All cell culture work was performed aseptically in accordance with good laboratory practice in an ABS optimale 18 laminar air flow unit (LAF). The LAF was allowed to run for at least twenty minutes prior to use, and sanitised using 70% industrial methylated spirits (IMS) in dH_20. All cell culture reagents were warmed in a water bath at 37°C for approximately thirty minutes prior to use, unless stated otherwise.

2.1.2 Prostate Cell Lines

Four prostate cancer cell lines were used in this project; PC-3, 22RV1, DU145 and LNCaP (Table 2.1). These cell lines were obtained from American Tissue Culture Collection (ATCC-LGC Standards, Teddington, Middlesex, UK). The PC-3 cell line was cultured in F12K Kaighn's Modified Medium (Biosciences, Ireland) supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, US) and 1% penicillin streptomycin (Sigma-Aldrich, St. Louis, MO, US) (P/S - 5000 U/mL penicillin, 5000 U/mL streptomycin). Both the 22RV1 and LNCaP cell lines were cultured in RPMI (Sigma-Aldrich, St. Louis, MO, US) which was supplemented with 10% (v/v) foetal bovine serum (FBS) and penicillin streptomycin (P/S - 5000 U/mL penicillin, 5000 U/mL streptomycin). The DU145 cell line was cultured in Eagle's Minimum Essential Medium (Sigma-Aldrich, St. Louis, Mo, US) supplemented with 10% (v/v) foetal bovine serum (FBS), penicillin streptomycin (P/S - 5000 U/mL penicillin, 5000 U/mL streptomycin) and 2mM L-glutamine. All cells were grown in a 5% CO_2 humidified atmosphere.

Table 2.1 *Cell line panel.*

Cell Line	Tissue	ATCC® Number	Androgen Sensitivity	Genetic Lesions
PC-3	Prostate; bone	ATCC® CRL-2505™	Insensitive	*PTEN* and *p53* deletion
22Rv1	Prostate	ATCC® HTB-81™	Sensitive	-
DU145	Prostate; brain	ATCC® CRL-1435™	Insensitive	Mutated *p53* and *p16*
LNCaP	Prostate; left supraclavicular lymph node	ATCC® CRL-1740™	Sensitive	Silent mutation of *p53*, frameshift mutation in *PTEN*

2.1.3 Cell Subculture

Cells were visualised each day using an inverted phase-contrasted Nikon microscope (Nikon Corp., Tokyo, Japan). Sub-culturing was performed when cell cultures reached 80-90% confluency. Cell culture medium was decanted and the cells were washed with 5 mL 1X PBS (Fisher Scientific, Dublin, Ireland) to remove residual FBS. Two mL of trypsin ethylene-diamine tetra-acetic acid (EDTA) (200 mg/mL Versene/EDTA), was added to the flasks. Flasks were incubated at 37°C for approximately 5 min to remove adherent cells from the surface. Eight mL complete medium was then added to the flasks to inactivate the trypsin. Cells were transferred to a sterile 15 mL tube and pelleted by centrifugation at 1300 g for 3 min (Centra GP8R, Thermo IEC). The supernatant was discarded and the cell pellet re-suspended in 10 mL complete medium. This suspension was used to seed fresh flasks at a number of different ratios.

2.1.4 Mycoplasma Testing

Cell lines used in this project were tested for mycoplasma infection every 6-9 months using the MycoAlert™ mycoplasma detection kit (Lonza Group Ltd., Basel, Switzerland). A 2mL cell sample was spun at 200g for 5 min. 100µl of the supernatant was cleared to a fresh tube. The MycoAlert™ reagent and the MycoAlert™ buffer were reconstituted and allowed to equilibrate for 15 min. 100µl of the MycoAlert™ reagent was then added to the sample and incubated for 5 min. The luminescence was then measured (Reading A). 100µl of the

MycoAlert™ substrate was then added to the sample and incubated for 10 minutes. The luminescence was then measured again (Reading B). A MycoAlert™ positive control is included in the kit and is not a source of mycoplasma infection. Mycoplasma infection was determined by calculating the ratio of the readings, reading B/reading A. <0.9 is negative for mycoplasma and >1.2 is positive for mycoplasma contamination.

2.1.5 Cell Counting

The number of cells per unit volume of suspension was estimated using a haemocytometer counting chamber. The haemocytometer was cleaned and a glass coverslip placed over both chambers. Cells were suspended in trypan blue vital stain (Fisher Scientific, Dublin, Ireland) and pipetted gently onto the edge of the coverslip such that the area under the glass coverslip was filled by capillary action. This procedure was repeated for the second chamber. The haemocytometer was visualised under 10X power and the number of live cells counted within the central 5x5 square of both chambers. The number of cells per ml volume was calculated as follows;

Total number of live cells/ml = $[(a+b)/2 \times 20 \times 10^4]$

Whereby a represents the first chamber, b represents the second chamber, 20 is the dilution factor and 10^4 is the conversion factor.

2.1.6 Cryopreservation

Cells were trypsinised as described in section 2.1.3 and a cell count was performed. 1 ml aliquots containing 1-1.5 x 10^6 cells/ml suspended in freezing solution were placed in 1.5 ml cryovials. Freezing solution was prepared freshly with complete media and 10% DMSO (Sigma Aldrich, St. Louis, MO, USA). Cryovials were stored at -80°C for 24 hours before being transported to liquid nitrogen for long term storage.

2.1.7 Propagation of Cells from Liquid Nitrogen Storage

Complete media was warmed to 37°C. Cells were removed from liquid nitrogen storage and thawed rapidly in a 37°C water bath for 2 min. Once thawed, the cells were added to 9 mls of complete media in a 15 ml tube. The cells were then spun at 300 g for 5 min. The supernatant was decanted and the cell pellet was resuspended in 1ml of warmed complete media. The cells were added to a tissue culture flask with the required volume of media. The cells were then incubated at 37°C with 5% CO_2.

2.1.8 Prostate Cancer Stem Cell Derivation

2.1.8.1 Holoclone Derivation using High-Salt Agar

A 1% molecular grade agarose (Sigma Aldrich, St. Louis, MO, USA) and NaCl (Sigma Aldrich, St. Louis, MO, USA) solution was prepared in dH_2O and subsequently autoclaved. 40 mls of the high-salt soft-agar mixture was added to petri dishes (Corning Incorporated, NY, USA) and allowed to solidify. Once the plates had set, 15 mls of the appropriate media was added. 1 x 10^6 cells were subsequently introduced to the plate and incubated at 37°C with 5% CO_2. 7-10mls of spent media was carefully removed by pipetting from the periphery of the plates every seven days and replaced with fresh, warmed media. Differential holoclone generation efficiencies were observed for each cell line examined (5.2.2), however at most 2-4 holoclones were observed in each plate at a time. Holoclones were maintained in culture until they reached maximum growth potential (as outlined in 5.2.2). It is noteworthy that this technique was highly inefficient as often no holoclones were observed across multiple agarose plates. The low-density culture of immortalised cells in a high-salt environment is postulated to select for robust cells with self-renewal potential (Olszewski *et al.*, 2005). Previous work performed within the laboratory has demonstrated that this technique successfully selects for a stem-like population in both ovarian and thyroid carcinoma cell lines (Sommerville *et al., in* preparation). The theory behind holoclone derivation as a surrogate technique for the identification of cancer stem cells is discussed in more detail in Chapter 5 (5.1.1-5.1.6).

2.1.8.2 Holoclone Derivation using MoFlo Single Cell Propagation/Colony Forming Assay

This technique is predicated on the basis that a single cell in culture will only yield a large, successful colony if it possesses the ability to self-renew; a defining characteristic of both normal and malignant stem cells. Cells were cultured and harvested as described in section 2.1.3 at 70-80% confluence and 5 x 10^5 cells were resuspended in sterile PBS + 1% BSA. A single cell was seeded into each well of a round-bottomed 96-well plate (Nunc, Germany) with a MOFLO flow cytometer (DakoCytomation). Two days following plating, 96-well plates were examined using microscopy. Wells containing only one viable cell were marked. Seven days following plating colonies were classified as holo-, mero-, and paraclones based on their morphologies and harvested fourteen days following flow sorting. Holoclones are postulated to contain self-renewing cancer stem cells, while paraclones are believed to constitute terminally differentiated cells. Meroclones are promulgated as intermediate colonies, which contain a dichotomy of stem-like and differentiated cells (described in further detail in Chapter 5).

2.1.8.3 Propagation of Spheres in Serum-free Stem Cell Medium

Prostatosphere growth was assessed through culture in serum –free stem cell medium supplemented with human growth factors. This technique has previously been demonstrated to yield cells of a stem phenotype (Duhagon *et al.,* 2010). Dulbecco's Modified Eagles Medium/Nutrient Mixture F-12 Ham (Sigma Aldrich, St. Louis, MO, USA) was supplemented with 0.4% BSA (Sigma Aldrich, St. Louis, MO, USA), 1% penicillin streptomycin (Sigma Aldrich, St. Louis, MO, USA), 2.5 mg insulin (Gibco®, Life Technologies, Biosciences, Ireland), 5 µg recombinant human fibroblast growth factor (FGF) (Cell Signalling, MA, USA), and 10 µg recombinant human epidermal growth factor (Peprotech, New Jersey, USA). Cells were seeded at low density (1 x 10^4) in a T25 flask and observed every 2-3 days for sphere formation. Cells were harvested as described in section 2.1.3.

2.1.9 Holoclone Harvesting

2.1.9.1 High Salt Agar

Each holoclone was gently removed from the agarose using a p20 pipette. The holoclones were placed into 1ml of complete media and spun at 300 g for 5 min to allow a pellet to form. The supernatant was removed carefully so as to avoid disturbing the pellet. Pellets were washed in cold 1X PBS and stored at -80 °C until needed.

2.1.10 96-well Plate

Spent media was removed from the well using a p100 pipette and the cells washed with 50 µl PBS. 50 µl of trypsin ethylene-diamine tetra-acetic acid (EDTA) (200 mg/mL Versene/EDTA) was added to the well and the plate incubated at 37 °C 5% CO_2 for 7 min. The cell and trypsin suspension was then removed and added to a sterile tube containing 200 µl warmed complete medium. The cells were spun at 300 g for 5 min to form a pellet. The supernatant was carefully removed and cell pellets stored at -80 °C.

2.2 Archival Tissue

2.2.1 Ethical Approval

Ethical approval in accordance with the Helsinki Declaration was obtained for this study from the Mater Misericordiae University Hospital, Beaumont Hospital and St. James's Hospital ethics committees. Radical prostatectomy samples were selected from the archival formalin-fixed paraffin-embedded (FFPE) tissue collected between the years 2006 and 2012 at these institutions. Slides had previously been reviewed by a pathologist and diagnoses confirmed according to the World Health Organisation classification guidelines (Hamilton *et al.,* 2000).

2.2.2 FFPE Sample Preparation

Formalin-fixed paraffin-embedded (FFPE) samples were cut at 7µm using a HM325 rotary microtome (MSC, Dublin, Ireland). The sections were floated in a 56°C waterbath and mounted onto slides. The sections were dried overnight by placing the slides on a heating block. The sections were subsequently deparaffinised and stained with haematoxylin and eosin (See Appendix 1.1).

2.3 RNA Isolation

2.3.1 RNA Isolation from FFPE Tissues

RNA was isolated from formalin-fixed paraffin-embedded tissues using the Ambion® RecoverAll™ Total Nucleic Acid Isolation Kit (Invitrogen Life Technologies, Maryland, and USA). Three cores (per patient) were removed from the dominant tumour nodule of paraffin blocks (as identified from freshly cut H&E-stained sections by the study pathologist), ground in liquid nitrogen and placed in an RNase/DNase-free eppendorf. In the case of tissue sections (Chapter 4), the area of interest was macrodissected from the slide using a scalpel and placed in an RNase/DNase-free eppendorf. 1ml of 100% xylene was added to the sample, and briefly vortexed. The sample was heated for 3 min at 50 °C to melt the paraffin. The sample was centrifuged for 2 min at room temperature and maximum speed to pellet the tissue. Xylene was removed from the sample and discarded. 1ml of 100% EtOH was added to the sample and centrifuged for 2 min at room temperature and maximum speed. The EtOH was removed and discarded and a second wash of 100% EtOH was performed. Residual EtOH was removed and the pellet allowed to air dry for 15-45 minutes at room temperature. 100µl of digestion buffer and 4µl of protease were added to each sample. The sample was incubated at 50°C for three hours. 120µl of isolation additive and 275µl of 100% EtOH were added to the sample, and mixed by pipetting up and down. 700µl of sample/ethanol mixture was added to a filter cartridge and centrifuged for 30s at 10,000 g to pass the mixture through the filter. This was repeated until all the sample mixture had passed through the filter. 700µl of wash buffer 1 was added to the filter cartridge and centrifuged for 30s at 10,000 g. The flow-through was discarded and 500µl of wash buffer 2 was added to the filter cartridge and centrifuged for 30s at 10,000 g. 4µl DNase, 6µl 10X DNase buffer and 50µl of nuclease-free water were added to eliminate DNA from the sample. The filter cartridge containing the DNase mix was incubated at room temperature for 30min. 700µl of wash buffer 1 was added and incubated for 30-60s at room temperature. The sample was spun at 10,000 g for 30s and the flow-through discarded. Two washes with 500µl wash buffer 2 were performed and the assembly centrifuged for a final 30s at 10,000 g

to remove residual fluid from the filter cartridge. 60µl elution solution was added to the centre of the filter and incubated for 1min at room temperature. The sample was centrifuged for 1min at maximum speed to pass the mixture through the filter. The eluate containing RNA was stored at -80°C in a sterile DNA LoBind tube to maximise downstream recovery (Eppendorf AG, Hamburg, Germany). Depending on the size of the tumour area isolated, RNA concentration was found to range from <2ng/ µl to >150ng/ µl. RNA samples for expression analysis were deemed suitable if concentration was found to be ≥7ng/µl. This cutoff was established to maximise the number of samples available for analysis. It was hypothesised that a preamplification step prior to analysis would circumvent any issues with low RNA input. The Nanodrop 260/280 ratio was utilised to ensure isolated RNA was free of contaminants and all RNA samples were found to be of sufficient quality (2.4.1).

2.3.2 RNA Isolation from Parental Cells and Holoclones

RNA was isolated from founder cells and holoclones using the RNeasy Mini Kit (Qiagen, West Crawley, Sussex, UK). Holoclones were harvested as in 2.1.9/2.1.9.1 and RNA extracted directly from the resultant pellet. Cells grown in suspension were pelleted by centrifugation for 5 min at 300 g and supernatant removed by aspiration. Cells were disrupted by the addition of 350µl buffer RLT, and vortexed to mix. Cells were further homogenized by vortexing for 5 min. One volume of 70% EtOH was added to the homogenized lysate and mixed well by pipetting. Up to 700µl of the sample was added to an RNeasy spin column and centrifuged for 15s at 8000 g. The flow-through was discarded. Genomic DNA contamination was eliminated as follows; 350µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15s at 8000 g, flow-through was discarded. 10µl DNase I solution was added to 70µl Buffer RDD and mixed by gently inverting the tube. The DNase I incubation mix was added directly to the RNeasy spin column membrane and incubated at room temperature for 15 min. Following the incubation, 350µl Buffer RW1 was added to the spin column and centrifuged for 15 s at 8000 g. 500µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at 8000 g and the flow-through discarded. An additional 500 µl Buffer RPE was added to the RNeasy column and centrifuged for 2 min at 8000 g to dry the membrane. The RNeasy column was then placed in a fresh 2 ml collection tube and the assembly centrifuged at full speed for 1 min to avoid any carry-over of Buffer RPE. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30-50 µl RNase-free H_2O was added directly to the spin column membrane. The assembly was centrifuged for 1 min at 8000 g to elute the RNA. The eluate containing RNA was stored at -80°C in a sterile DNA LoBind tube to maximise downstream recovery (Eppendorf AG, Hamburg, Germany).

2.3.3 RNA Isolation from Fresh Frozen Tissue

RNA was isolated from fresh frozen tissue sections using the RNeasy Mini Kit (Qiagen, West Crawley, Sussex, UK). Up to 8.5 micron sections were cut using a cryotome and placed in a 2 ml microcentrifuge tube containing a stainless steel bead. The tissue was disrupted and homogenized using the *TissueLyser* (Qiagen, Crawley, West Sussex, UK) as follows; 600 µl Buffer RLT was added to the microcentrifuge tube. The tube was placed in the TissueLyser Adapter Set 2 x 24 and run for 2 min at 20 – 30 Hz. The resultant lysate was then centrifuged for 3 min at full speed. The supernatant was carefully removed and transferred to a new microcentrifuge tube. Only the supernatant (lysate) was used in subsequent steps. One volume of 70% EtOH was added to the cleared lysate and mixed immediately by pipetting. 700 µl of the sample was transferred to an RNeasy spin column and centrifuged for 15 s at 8000 g. The flow-through was discarded. Elimination of genomic DNA contamination was performed as in 2.2.2. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at 8000 g and the flow-through discarded. An additional 500 µl Buffer RPE was added to the spin column and centrifuged for 2 min at 8000 g to wash the membrane. The RNeasy spin column was then placed in a new 2 ml collection tube and the assembly was centrifuged at full speed for 1 min to avoid any carry-over of buffer RPE. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30-50 µl RNase-free H_2O added directly to the membrane. The assembly was centrifuged for 1 min at 8000 g to elute the RNA. The eluate containing RNA was stored at -80°C in a sterile DNA LoBind tube to maximise downstream recovery (Eppendorf AG, Hamburg, Germany).

2.4 RNA Quantification

2.4.1 RNA Quantification using the Nanodrop

RNA was quantified using a NanoDrop 1000 spectrophotometer (version 3.1.0, Nanodrop Technologies). The instrument was first initialised and blanked with 1 µL of RNase/DNase-free H_2O. One µL of each sample was loaded individually onto the NanoDrop. The instrument was cleaned between each sample. The NanoDrop returned the nucleic acid concentration in ng/µL and also the 260:280 and 260:230 purity ratios.

2.4.2 RNA Quantification using the Qubit® Fluorometer

Quant-iT™ working solution was prepared by adding 1 x n µl Quant-iT™ reagent to n x 199µl Quant-iT™ buffer (where n = number of standards plus number of samples). Standard 1 was prepared by adding 190 µl of the working solution to 10 µl of kit standard 1. Standard 2 was prepared by adding 190 µl of the working solution to 10 µl kit standard 2. Samples

were prepared for quantification by adding 198 µl working solution to 2 µl RNA sample to be quantified. All assay tubes were vortexed for 2-3 seconds and incubated at room temperature for 2 min. The samples were read in the Qubit® Fluorometer (Life Technologies, Biosciences, Dublin, Ireland) and the resultant reading multiplied by the dilution factor to determine concentration of original RNA sample. The Qubit® Fluorometer was used in addition to the Nanodrop as the Qubit® provides a more accurate quantification of RNA at lower concentration. It is also prudent to note that the Qubit gives no qualitative measure while the Nanodrop method indicates the presence of potential contaminants in the RNA sample. As such, both techniques were utilised.

2.5 miRNA and Gene Expression Analysis

2.5.1 cDNA Synthesis for Gene Expression Analysis

cDNA was synthesised using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) by the addition of the following components into a sterile, nuclease-free tube on ice; up to 2 µg total RNA (as isolated in section 2.2), 2 µl of 10X RT Buffer, 0.8 µl 25X dNTP Mix, 2.0 µl RT Random Primers, 1.0 µl Multiscribe Reverse Transcriptase, 1.0 µl RNase Inhibitor and 3.2 µl Nuclease-free H_2O to a final volume of 20 µl. The thermal cycling conditions were as follows; 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C and hold at 4 °C. cDNA was stored at -20 °C until use.

2.5.2 cDNA Synthesis for miRNA Expression Analysis

cDNA for use in miRNA expression analysis was synthesised using the TaqMan© miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) by the addition of the following components into a sterile, nuclease-free tube on ice; 5 µl total RNA (as isolated in 2.3), 0.15 µl 100mM dNTPs, 3 µl 5X RT Primer, 1 µl Multiscribe Reverse Transcriptase, 1.5 µl 10X RT Buffer, 0.19 µl RNase Inhibitor, 4.16 µl of Nuclease-free H_2O to a final volume of 15 µl. The thermal cycling conditions were as follows; 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C, hold at 4 °C. cDNA was stored at -20 °C until use.

2.5.3 TaqMan® Probe-Based Gene/miRNA Expression Analysis

cDNA generated using the protocols described in 2.5.1 and 2.5.2 was used for gene expression analysis using TaqMan® chemistry. Each PCR reaction was prepared in a 96-well plate as follows; 1.0 µl 20X TaqMan® gene expression assay (in the case of miRNA expression analysis 1.0 µl 20X TaqMan® small RNA assay), 10.0 µl TaqMan® Universal Master Mix, 4 µl cDNA template (1 to 100 ng), 5.0 µl RNase-free H_2O to a final volume of 20 µl. The negative control for each reaction consisted of 1 µL RNase-free H_2O substituted for

template cDNA. The plates were loaded onto the 7500 Fast Real-Time PCR Machine (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. The comparative C_T method was used to analyse the results of gene and miRNA expression analysis using the DataAssist Software (Applied Biosystems, Foster City, CA, USA).

2.5.4 TaqMan® Probe-Based miRNA Expression Analysis using Preamplification

A miRNA reverse transcription primer pool was constructed by adding 10 µl of each 5X RT primer to a sterile nuclease-free tube and brought to a final volume of 1000 µl using 1X TE Buffer (Sigma Aldrich, St. Louis, MO, USA). The RT reaction mix was prepared as follows; 3 µl total RNA sample, 6 µl RT Primer Pool, 0.30 µl 100mM dNTPs, 3 µl Multiscribe Reverse Transcriptase, 1.5 µl 10X RT Buffer, 0.19 µl RNase Inhibitor and 1.01 µl Nuclease-free H_2O to a final volume of 15 µl. The thermal cycling conditions were as follows; 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C, hold at 4 °C. A PreAmp primer pool was constructed by adding 10 µl of each 20X TaqMan® MicroRNA Assay to a sterile nuclease-free tube and brought to a final volume of 1000 µl using 1X TE Buffer. The preamplifcation reaction mix was prepared as follows; 2.5 µl RT Product, 12.50 µl TaqMan PreAmp Master Mix, 3.75 µl PreAmp Primer Pool, 6.25 µl Nuclease-free H_2O to a final volume of 25 µl. The preamplification thermal cycling conditions were as follows; 10 min at 95 °C, 2 min at 55 ° C, 2 min at 72 °C, 12 cycles of 15 sec at 95 °C and 4 min at 60 ° C, a final step of 10 min at 99.9 °C and hold at 4 °C. 175 µl of 0.1X TE buffer was subsequently added to each reaction. The diluted preamplification products could be stored at -20 °C for up to one week. The subsequent PCR reaction mix was prepared as follows; 1 µl 20X TaqMan® MicroRNA Assay, 0.20 µl diluted preamplification product, 10 µl Taqman® Universal Master Mix II, No AmpErase® UNG, 8.80 µl Nuclease-free H_2O to a final volume of 20 µl. The thermal cycling conditions were as follows; 10 min at 95 °C, and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Data was analysed using the same protocol described in 2.5.3.

Table 2.2 *TaqMan® Gene Expression Assays.*

Gene Symbol	Gene Name	TaqMan® Assay ID	Notes
ALDH1A1	Aldehyde dehydrogenase	Hs000946916_m1	
SOX2	SRY-box2	Hs01053049_s1	
POUF51	POU class 5	Hs00999634_gH	AKA OCT4A

	homeobox1		
NANOG	Nanog Homeobox	Hs04260366_g1	
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase	Hs02758991_g1	Endogenous Control
CD44	CD44 molecule	Hs01075861_m1	
PROM1	Prominin 1/CD133	Hs01009250_m1	
ITGB1	Integrin beta 1	Hs01009250_m1	
ITGA2	Integrin alpha 2	Hs00158127_m1	
MET	Met proto-oncogene	Hs01565584_m1	
CD24	CD24 molecule	Hs00273561_s1	
hsa-miR-15a	microRNA 15a	000389	
hsa-miR-21	microRNA 21	000397	
hsa-miR16-1'	microRNA 16-1'	002420	
hsa-miR-125b	microRNA 125b	000449	
hsa-miR-20a	microRNA 20a	000580	
hsa-miR-34a	microRNA 34a	000426	
hsa-miR-222	microRNA 222	002276	
hsa-miR-221	microRNA 221	000524	
hsa-miR126	microRNA 126	002228	
RNU24	Small nucleolar RNA C/D box 24	001001	Endogenous Control
hsa-miR-331	microRNA 331	000545	
hsa-miR-200b	microRNA 200b	002251	
hsa-miR-101	microRNA 101	002253	

hsa-miR-146a	microRNA 146a	000468
hsa-miR-141	microRNA 141	000463
hsa-miR-330	microRNA 330	000544

2.6 Custom TaqMan® Low Density Arrays (TLDAs) with Preamplification

TaqMan® gene and miRNA low density 384-well arrays were performed according to manufacturer's guidelines available in Appendix 1. Briefly, arrays were designed with custom hybridised probes using the applied biosystems webpage (table 2.3).

http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-probe-based-gene-expression-analysis/taqman-gene-expression-assay-selection-guide.html

cDNA was synthesised as described in section 2.5. The subsequent preamplification reaction was prepared by adding 2 µl cDNA, 2 µl custom TaqMan PreAmp pool and 2 µl TaqMan PreAmp master mix (2X) to a nuclease-free PCR tube. The cycling conditions were as follows; 10 min at 95 °C, 15 sec at 95 °C, 4 min at 60 °C for 14 cycles, 10 min at 99.9 °C and hold at 4 °C. The PCR amplification reaction was prepared for each sample by adding 3 µl PreAmp product, 52 µl nuclease-free H_2O and 55 µl Taqman Universal Master Mix II, No AmpErase® UNG to a nuclease-free tube. 100 µl of this reaction mix was loaded into each reservoir of the TaqMan array microfluidic card (1:32 dilution of the preamplified cDNA). The TaqMan array was centrifuged for 2 min at 1200 g and subsequently sealed and trimmed. qPCR was performed on the TaqMan array microfluidic card. In the case of miRNA, the preamplification reaction was prepared by adding 12.5 µl TaqMan PreAmp master mix (2X), 3.75 µl custom TaqMan PreAmp pool, 6.25 µl nuclease-free H_2O, and 2.5 µl of RT product to a nuclease-free PCR tube. The thermocycling conditions were as follows 10 min at 95 °C, 2 min at 55 °C and 2 min at 72 °C, 15 sec at 95 °C and 4 min at 60 °C for 12 cycles, 10 min at 99.9 °C and hold at 4 °C. The preamplified cDNA was diluted to a final volume of 200 µl by the addition of 175 µl nuclease-free 0.1X TE buffer. The custom TaqMan miRNA array was prepared by adding 56.25 µl Taqman Universal Master Mix II, No AmpErase® UNG, 1.13 µl diluted preamp product and 55.12 µl nuclease-free H_2O to a nuclease-free tube. 100 µl of this reaction mixture was added to the appropriate reservoir and the plate was prepared as described for gene expression analysis.

Table 2.3 *TaqMan® Low Density Array Formats.*

	Array Format	Fill Reservoirs/sample	Targets/card	Samples/card	Ref. Code
Gene	12	1	12	8	4342247
miRNA	16	1	16	8	4449136

2.7 qRT-PCR Data Analysis

The $\Delta\Delta C_T$ method was used to calculate the relative expression of gene/miRNA targets. The ΔC_T value was calculated by subtracting the average C_T value (C_T: the cycle value at which the amplified target gene crosses a threshold level) of the endogenous control from the average C_T of the target gene. The $\Delta\Delta C_T$ value is obtained by subtracting the ΔC_T value of the chosen calibrator/reference sample (untreated control sample) from the ΔC_T value of each sample. The quantity of target gene expression relative to the calibrator sample (RQ value) is calculated using the formula $2^{-\Delta\Delta CT}$.

2.8 Flow Cytometry

Cells were trypsinised and counted as described in sections 2.1.3 and 2.1.5. 5 x 10^5 cells were resuspended in complete medium and spun for 3 min at 500g. The supernatant was removed and the cell pellet was resuspended in 100 µl PBA (1X PBS + 0.1 % NaAzide + 0.1% BSA). The appropriate amount of antibody (table 2.3) was added and subsequently incubated in the dark for 20 min, after which time 100 µl of PBA was added. The cells were then spun for 5 min at 300 g to remove any unbound antibody. The supernatant was then removed and the cells resuspended in 200 µl PBA. The samples were then acquired on a Dako CyAn ADP (Beckman Coulter, United States).

Table 2.4 *Antibodies used for flow cytometry.*

Antibody Name	Company	Ref. Code	Optimal Dilution
APC Mouse Anti-Human CD133	Miltenyi Biotec	130-090-853	1ul (neat)
PE-Cy™7 Mouse Anti-Human CD44	BD Biosciences	560533	1:2
PE Mouse Anti-Human CD29	BD Biosciences	557332	1:5
FITC-Mouse Anti-Human CD49b	BD Biosciences	555498	1:10

2.9 Tissue Microarray (TMA) Construction

A tissue microarray map was designed in order to create a guideline for the precise location of patient and control specimens. Control cores were placed whereby their position created uneven edges so the block could be correctly aligned once sections had been cut. A recipient block was made using a routine cassette, a blank mould and paraffin. Prior to use, all excess paraffin was removed from the cassette. The punches were placed into the tissue chip arrayer (Beecher Instruments, Silver Spring, MD USA) according to manufacturer's guidelines. The recipient punch (red) was inserted into the left-hand side of the arrayer and the donor punch (blue) was inserted into the right-hand side of the arrayer. Screwdrivers were used to tighten the punches and ensure correct alignment. The recipient block was placed into a slot and tightened until it could no longer be moved by hand. The TMA was started approximately 3mm from the edge of the recipient block to prevent breakage of the paraffin. Using the micrometers on the x and y axes, the punches were aligned into the start position. The red punch was gently pushed down into the recipient block. The lever was moved left to right 2-4 times. Holding the block steady, the punch was gently pulled upwards. The gold pin was pressed down to expel the wax from the punch. A small black pedestal was then placed over the recipient block onto which the patient donor block and the corresponding H&E section could be placed. The H&E stained slide (reviewed and marked by a pathologist) was aligned with the block until the correct position of the tumour was identified. The slide was then removed and the blue punch was gently pressed down into the tissue until resistance was met. The lever was once again moved from left to right and the

donor block was gently removed from the punch. The black pedestal was then removed and the blue punch was aligned 2-3mm above the hole created by the red punch. The gold pin was then gently pressed until the tissue emerged from the punch and into the recipient block. The tissue was left slightly exposed from the recipient block. A glass slide was gently pressed onto the core so that it became flush with the surface of the donor block. Once completed, the tissue microarray was placed between two glass slides and baked at 65 °C for approximately 5 min. The TMA and glass slides were then removed from the oven and placed on ice. After 2-3 min the glass slides were very gently removed to prevent accidental removal of cores. The tissue microarray block was then cut into 5µm sections on pre-treated slides to promote adhesion of the tissue section. The slides were baked overnight at 65 °C for immunohistochemistry.

2.9.1 Immunohistochemistry on the Roche Ventana Discovery XT Automated Platform

5 µm tissue sections were cut onto charged slides and baked overnight at 65 °C as described in section 2.2. A protocol was designed on the Discovery XT machine (Roche Ventana, Arizona, USA) according to optimum antibody conditions. Unique barcoded labels corresponding to the specific protocol were created and attached to the charged slides. The slides were inserted into the auto-stainer along with the required reagents. Upon protocol completion the slides were removed and washed with Dako® 1X wash buffer and distilled water to remove residual reagents. The slides were then placed in haematoxylin for 5-6 seconds and subsequently rinsed in running water to remove excess dye. The slides were then placed in 70% EtOH for 1 min, and 100% EtOH for 1-2 mins. The slides were then washed in xylene for 2 minutes and coverslipped for analysis. A number of routine staining procedures were also performed on the Dako Autostainer (Dako, Agilent, CA, USA).

Table 2.5 *Primary Antibodies.*

1° Antibody Name	Incubation Time	Optimal Dilution	Company	Ref. Code
Anti-ERG Rabbit mAB	32 min	Neat	Roche	EPR3864
Anti-Ki-67 Mouse mAB	32 min	1:80	Dako	IS62630-2
Anti-CCND1 Rabbit mAB	24 min	Neat	Dako	IS08330-2
Anti-Cytokeratin (Cam5.2) Rabbit mAB	16 min	Neat	Roche	790-4555
Anti-E-cadherin Mouse mAB	32 min	1:50	Invitrogen	18-0223
Anti-CD34 Mouse mAB	32 min	1:50	Dako	IR63261-2
Anti-Vimentin Mouse mAB	16 min	Neat	Dako	IR63061-2

Table 2.6 *Positive and Negative Controls for each Antibody.*

1° Antibody Name	Positive Control	Negative Control
Anti-ERG Rabbit mAB	Known TMPRSS2-ERG positive tissue/internal control: endothelial cells	Primary Ab Omitted
Anti-Ki-67 Mouse mAB	Human Skin	Primary Ab Omitted
Anti-CCND1 Rabbit mAB	Human Lung Tissue	Primary Ab Omitted
Anti-Cytokeratin (Cam5.2) Rabbit mAB	Human Skin	Primary Ab Omitted
Anti-E-cadherin Mouse mAB	Human Breast Carcinoma	Primary Ab Omitted
Anti-CD34 Mouse mAB	Human Bone Marrow	Primary Ab Omitted
Anti-Vimentin Mouse mAB	Human Placenta	Primary Ab Omitted

Table 2.7 *Secondary Antibodies.*

2° Antibody Name	Incubation Time	Optimal Dilution	Company	Ref. Code
OmniMap anti-Rabbit HRP	20 min	As per SOP	Roche	760-4311
Universal Secondary Antibody MultiLink	20 min	As per SOP	Dako	E045301-2

2.9.2 Statistical Analysis of Tissue Microarray Data

Tissue microarray slides were scored by a pathologist (as described in 3.2.6) and resultant data was analysed using the statistical analysis software SAS (Cary, North Carolina, USA) by a Harvard Collaborator (Dr. Irene Shui). The Chi-Squared test was used to analyse the relationship between variables. Logistic regression analysis was also performed to examine the relationship between protein expression and clinicopathological characteristics.

2.10 Feasibility Xenotransplantation Assay

PC-3 parental and holoclone cells were dissociated as described in 2.1.3 and 2.1.10. The cells were counted and in a 1.5 ml eppendorf tube, serially diluted to the desired concentration of 5000 cells in 400 µl of ice cold Ham's F12 media (Sigma-Aldrich, St. Louis, MO, USA). 100 µl of matrigel (BD Biosciences, Dublin, Ireland), was added to this 400 µl cell suspension. This was performed using an ice-cold pipette tip. Matrigel was thawed overnight at 4 °C prior to use. The injection site on the mice was shaved the day prior to injection and ear punches were applied in order to identify the mice. 100 µl of matrigel/cell suspension was injected subcutaneously above the right hind-limb of NOD/SCID mice (n=4 parental, n=4 holoclone). Once injected, the needle was held in place for 5 seconds before withdrawal in order to prevent cell suspension flowing back out of the injection site. When derivative tumours had reached the predefined ethical limit of 1 cm diameter, mice were euthanized by cervical dislocation followed by CO_2 asphyxiation to confirm death. A portion of the harvested tumours was snap-frozen in liquid N_2 and stored at -80 °C for later use. The remainder of the tumour was histologically prepared for analysis by a pathologist. Tissue was fixed using 10% neutral buffered formalin (NBF) for no more than 8 hours. Tissue was then placed in

cassettes and dehydrated using sequential EtOH baths (30%, 50%, 70%, 80%, 90%, 95% and 100%). Prior to paraffin embedding tissue was cleared through soaking in 50:50 toluene and alcohol. Tissue was then paraffin embedded and sections were cut and H&E-stained for review by the pathologist.

2.10.1 Validation Xenotransplantation Assay

PC-3 and DU145 parental and holoclone cells were dissociated as described in 2.1.3 and 2.1.10. The cells were counted and in a 1.5 ml eppendorf tube, serially diluted to the desired concentration of 20,000 cells in 500 µl of ice cold 1X PBS (Fisher Scientific, Dublin, Ireland). Each 100 µl injection comprised of 50 µl cell suspension (~3000 cells) and 50 µl ice cold Matrigel (BD Biosciences, Dublin, Ireland). As in 2.10, matrigel was thawed at 4 °C overnight prior to use. Cells were injected subcutaneously into the flanks of NOD/SCID mice. When derivative tumours had reached the predefined ethical limit of 2 cm diameter, mice were euthanized by cervical dislocation. A portion of the harvested tumours was snap-frozen in liquid N_2 and stored at -80 °C for later use. The remainder of the tumour was histologically prepared for analysis as described in 2.10.

2.11 Next-generation Sequencing on the Illumina® HiSeq® 2500 Platform

Next-generation sequencing of small (single read) and long ncRNA (paired-end) repertoires of clonally-derived holoclones and their derivative tumour xenografts was performed by Clinical Genomics (Mount Sinai Hospital, Toronto, Canada) using the Illumina® HiSeq® 2500 platform (Illumina, CA, USA). Illumina's sequencing technology, SBS (sequencing by synthesis) is the most successful and broadly utilised next-generation sequencing technology in the world (Quail et al., 2012). The Illumina® Cluster Station® isothermally amplifies DNA on a flow cell surface to generate clusters, each of which contains 500-1000 clonal copies of a single template molecule. The flow cell surface is coated with oligonucleotides, which correspond to the sequences of the adaptors ligated during the library preparation stage (Figure 2.1). The high-density clonally-amplified array of templates, which is immobilised to an acrylamide coating on the flow cell surface, then undergoes sequencing by synthesis using fluorescently-labelled reversible terminator nucleotides (Figure 2.2). In the case of paired-end reads, following completion of the first read, the clusters are modified to regenerate the template for the paired read. The same clusters are then sequenced using a second primer to generate the second read. The benefit of utilising paired-end reads for sequencing of mRNA and long ncRNAs is that the paired end nature allows more accurate alignments.

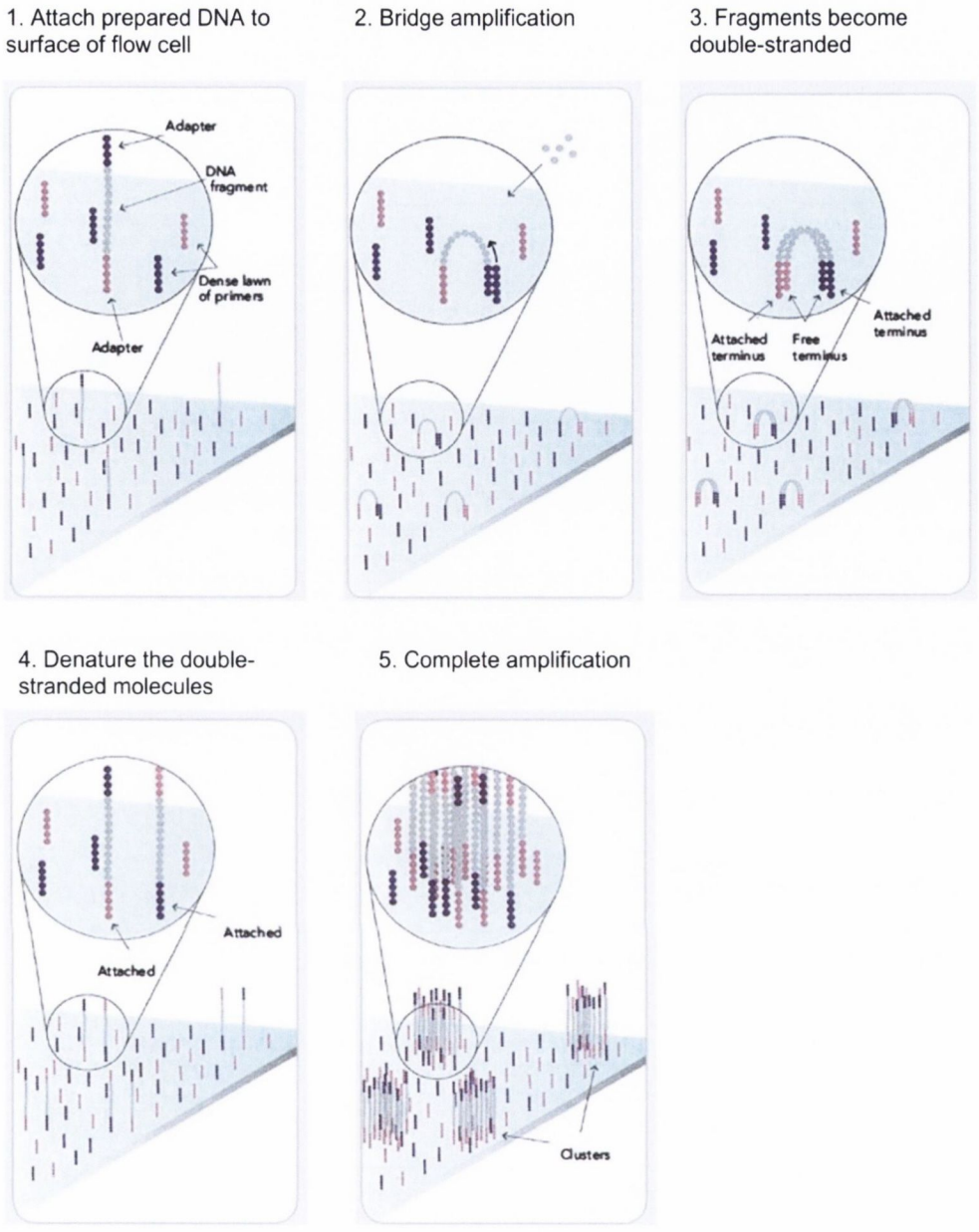

Figure 2.1 *Cluster generation by bridge amplification.*

1. Single-stranded fragments are randomly bound to the inside surface of the flow cell channels. 2. Unlabelled nucleotides and enzyme are added to initiate solid-phase bridge amplification. 3. The enzyme incorporates nucleotides to build double-stranded bridges on the solid phase substrate. 4. Denaturation leaves the single-stranded templates immobilised on the substrate. 5. Each flow cell of the channel contains several million clusters of double-stranded DNA. Adapted from Illumina Tech Summary.
http://seqanswers.com/forums/images/content/ilmn-step1-6.jpg

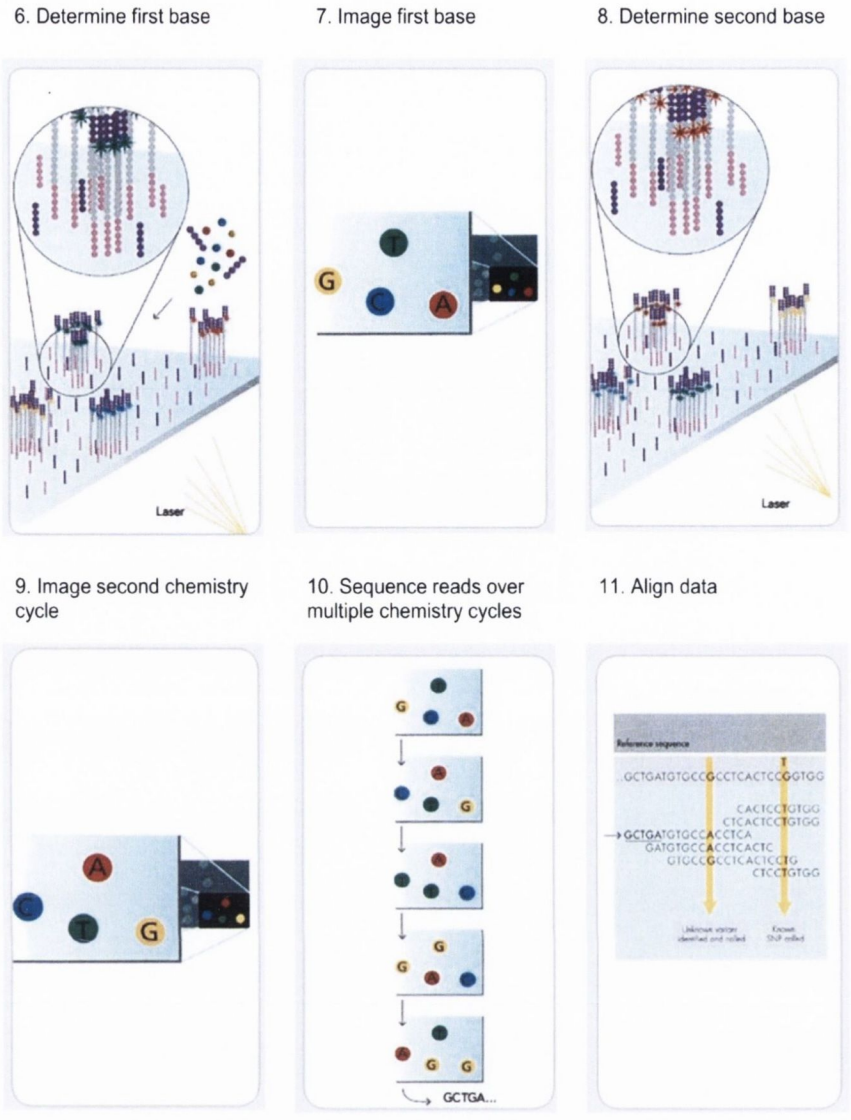

Figure 2.2 *Principle of sequencing by synthesis.*

6. In order to initiate the first sequencing cycle, all four labelled reversible terminators, primers and DNA polymerase are added to the flow cell. 7. Following laser excitation, the fluorescence emitted from each cluster on the flow cell is captured. The identity of the first base is recorded for each cluster. 8. To initiate the next sequencing cycle, all four labelled, reversible terminators and enzyme are added to the flow cell. 9. Following laser excitation, the image data is captured as before. The identity of the second base of each cluster is identified. 10. The cycles of sequencing are repeated to determine the sequence of bases in a given fragment. 11. Data is aligned, compared to a reference genome/transcriptome and sequence differences are identified.

Adapted from http://seqanswers.com/forums/images/content/ilmn-step1-6.jpg.

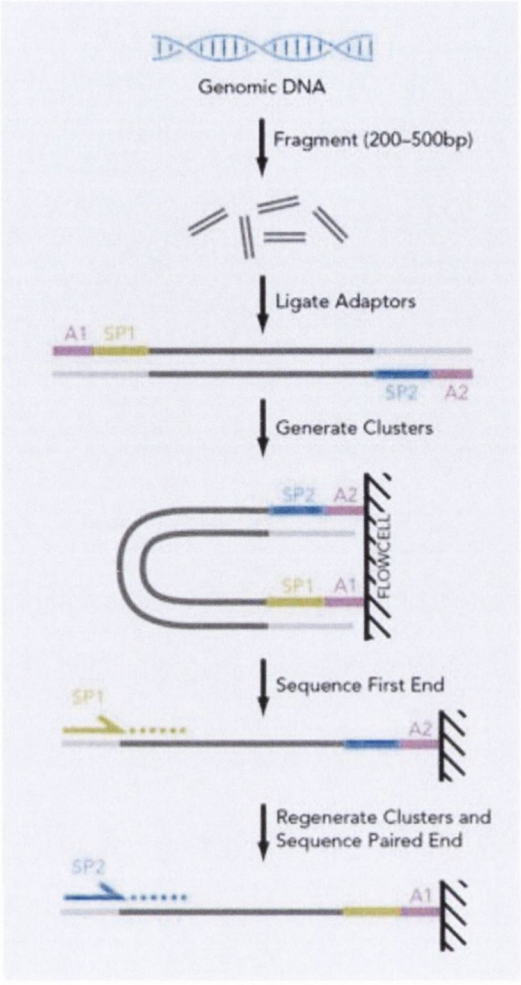

Figure 2.3 *Paired-end sequencing.*

This technique allows both ends of the fragment to be sequenced. As the distance between each paired read is known, alignment algorithms can utilise this information to more accurately map the reads, particularly over repetitive regions of the genome. A typical paired-end run can achieve 2 x 75bp reads and up to 200 million reads. Paired-end sequencing requires the same amount of input gDNA or cDNA as single-read sequencing. Adapted from www.illumina.com.

2.11.1 Protein-coding and Long ncRNA Library Preparation using the TruSeq® Stranded Total RNA Sample Prep Kit

Total RNA samples were prepared for sequencing of mRNA and long ncRNA as per the manufacturer's instructions. For full protocol see Appendix I.

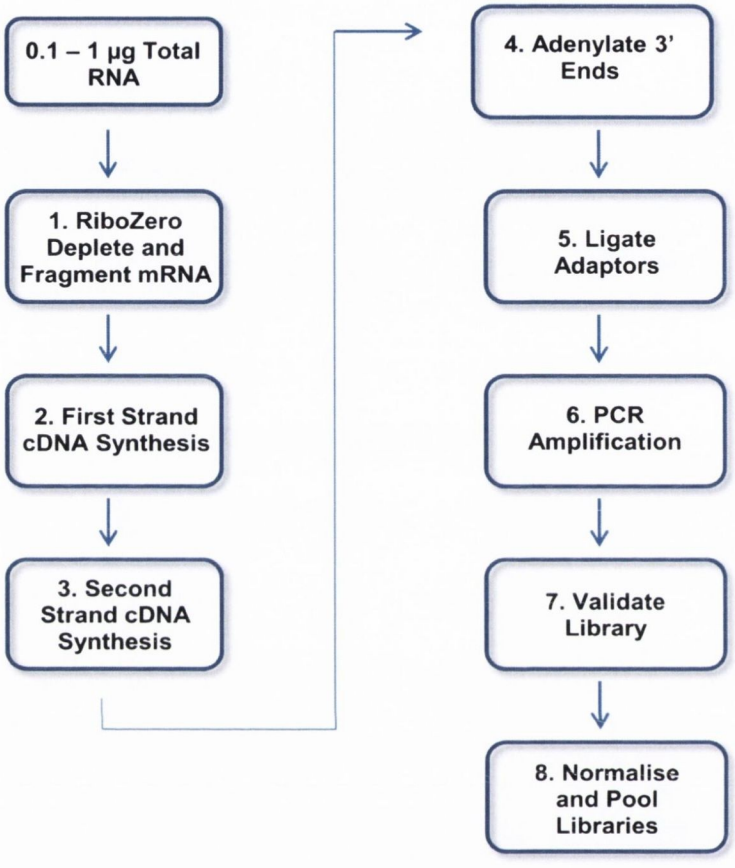

Figure 2.4 *Workflow for Illumina® TruSeq® Long ncRNA library preparation.*

1. rRNA is depleted from total RNA. Following depletion the remaining RNA is purified, fragmented and primed for cDNA synthesis. 2. The cleaved RNA fragments are reverse transcribed with random hexamers into first strand cDNA. 3. The template RNA is removed and a replacement strand is synthesised to generate dsDNA. 4. A single 'A' is added to the 3' end of the blunt fragments in order to prevent self-ligation during adaptor ligation. 5. Multiple indexing adaptors are ligated to the ends of the dsDNA. 6. PCR is used to selectively enrich DNA fragments that have adaptor molecules on both ends. This amplifies the amount of DNA in the library. 7. Library size and purity is validated using the Agilent Technologies 2100 Bioanalyser. 8. DNA templates are prepared for cluster generation.

2.11.2 Small ncRNA Library Preparation using the Bioo Scientific® NEXTflex™ Small RNA Library Prep Kit

Total RNA was prepared for sequencing of small ncRNA using the Bioo Scientific® NEXTflex™ Small RNA Library Prep Kit as per manufacturer's instructions. For full protocol see Appendix I.

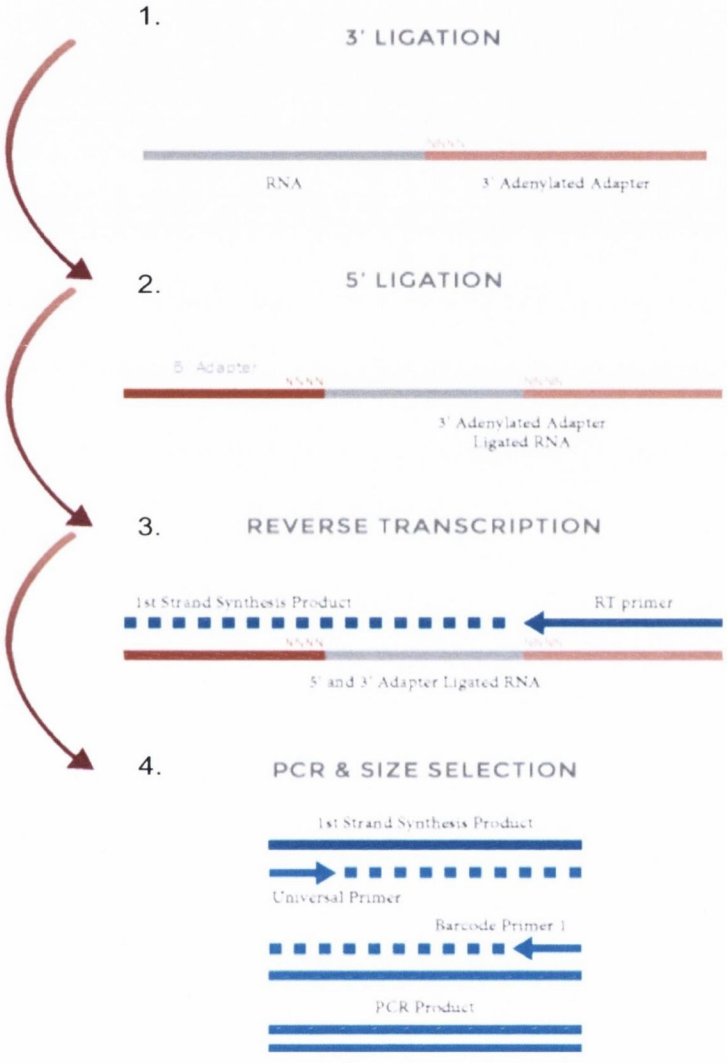

Figure 2.5 *Small RNA sample preparation flow chart.*

1. 3' NEXTflex™ 4N adenylated adaptor ligation and excess 3' adaptor removal. 2. 5' NEXTflex™ 4N adenylated adaptor ligation. 3. Reverse transcription and first strand synthesis followed by bead cleanup step. 4. PCR amplification and subsequent gel electrophoresis. Libraries are size selected from the agarose gel band and purified. Library size and purity is validated using the Agilent Technologies 2100 Bioanalyser. Adapted from Bioo Scientific® NEXTflex™ Small RNA Library Prep Kit Protocol (Appendix 1.18).

2.11.3 Analysis of Sequencing Data

Reads generated on the Illumina® HiSeq platform were quality-trimmed using Cutadapt and Sickle (Martin, 2011; Joshi *et al.,* 2011). Filtered reads were mapped to the Ensembl Human reference genome (build GRCh38, release 76). Short RNAs were mapped using Bowtie, while long RNAs were mapped using the splice-aware aligner Tophat, allowing reads that span splice junctions to be properly mapped (Langmead *et al.,* 2009; Trapnell *et al.,* 2009). Read counts were calculated across all transcripts using HTSeq (Anders *et al.,* 2014). Transcripts which received low average counts (≤100) across samples were excluded, to promote evidence-based results. Differential expression analysis was performed using edger (Robinson *et al.,* 2010). Log_2 fold change was calculated internally by edgeR.

miRNA Expression Profiling in an Archival Prostate Cancer Cohort

Chapter 3

Chapter 3. miRNA Expression Profiling in an Archival Prostate Cancer Cohort

3.1 Introduction

3.1.1 microRNAs: Potential Biomarkers in Prostate Cancer

The seminal discovery that miR-15a and miR-16-1 deletions are implicated in the development of chronic lymphocytic leukaemia has revolutionised our understanding of the pathogenesis of human malignancies (Calin *et al.,* 2002). For many years, it was widely understood that tumours were predominantly initiated in a multi-step process by somatic genetic alterations and epigenetic mutations to protein-coding genes. However, the identification of small, regulatory RNAs and the elucidation of their role in human disease has drastically altered our perception of tumorigenesis and provided a desirable means to further understand the events that initiate cancer development.

Prostate cancer is the most widely diagnosed cancer (Peng *et al.,* 2011). It is estimated as the second leading cause of cancer-related mortality in men greater than 40 years old in the United States (Jemal *et al.,* 2010). The intrinsically heterogeneous nature of this malignancy is one of the major confounding factors in not only understanding but also successfully treating prostate cancer. The overall natural history of this disease is relatively favourable. The five-year survival rates of early-stage prostate cancer are 99% (Eastham *et al.,* 1998). However, in stark contrast, 46% of patients with metastatic disease will die within 22 months of receiving diagnosis (DeMarzo *et al.,* 2007). As outlined in Chapter 1, there remains no reliable method to distinguish those men with the greatest risk of disease progression. Hence, there is an urgent requirement for secondary diagnostic and prognostic biomarkers as an adjunct to PSA testing and Gleason grading. The implication of widespread miRNA dysregulation in the initiation and perpetuation of human malignancy has raised the question whether differential miRNA expression profiles may be harnessed to construct a molecular signature of aggressive prostate cancer. As previously mentioned in section 1.7.3, the well-documented stability of miRNAs supports their implementation as minimally invasive, robust biomarkers. Furthermore, the tissue-specific nature of miRNAs facilitates their use in the diagnosis of cancer of unknown primary site and tumour subtype classification. This chapter will focus on a number of miRNA candidates whose aberrant expression has been established as biologically relevant in the development of prostate cancer and examine whether their expression profiles are distinct to aggressive disease in a small (n=50), archival cohort (Figure 3.1). The prostatectomy specimens were classified as putatively clinically indolent or aggressive based on a modified version of Epstein's criteria for the identification of clinically insignificant disease, and each patient group consisted of treatment-naive radical prostatectomy specimens excised between 2007 and 2012 (Epstein

et al., 1994). The crucial issue we sought to investigate was whether a differential miRNA expression profile exists, which may distinguish patients with intrinsically aggressive prostate neoplasms.

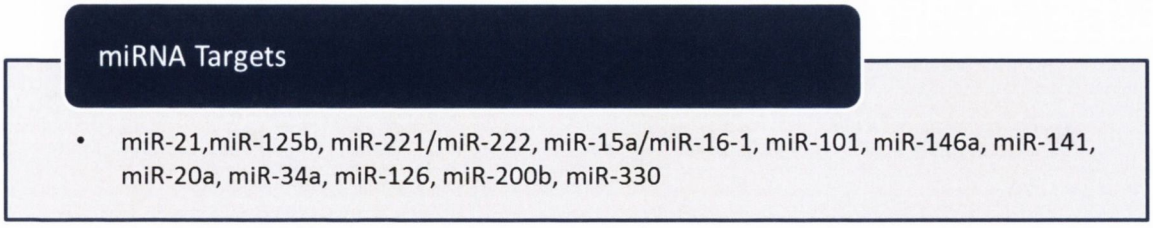

Figure 3.1 *MicroRNA panel for investigation*

This miRNA panel was identified through a meta-analysis of the literature in order to identify the most biologically relevant differentially expressed miRNAs in prostate carcinoma.

3.1.2 microRNA Dysregulation in Prostate Cancer

The expression patterns of many miRNAs have been described as dysregulated in prostate cancer; however the pathologic consequences of abnormal expression have only been elucidated and experimentally validated for a small number of miRNAs (Figure 3.2). These miRNAs are classified as either tumour suppressor miRs or oncomiRs in that they function to silence the expression of oncogenic genes or tumour suppressive genes respectively (Figure 3.3).

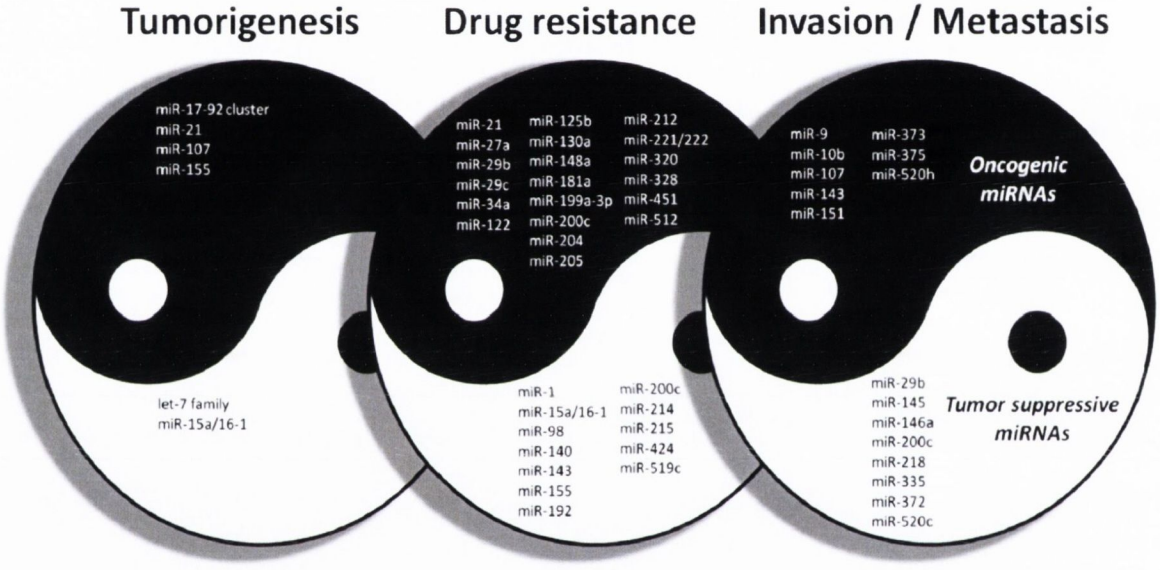

Figure 3.2 *miRNAs involved in cancer progression.*

A schematic depicting the miRNAs whose biological role in tumorigenesis has been characterised. These miRNAs are classified as either oncogenic or tumour suppressive miRNAs. Adapted from Chen *et al.*, (2012).

MicroRNA dysregulation	Targets	Consequences
MicroRNA overexpression	Tumour suppressors	↓ for example PTEN, p22, p57, TIMP3 and PDCD4.
MicroRNA loss	Oncogenes	↑ for example BCL2, MCL1, RAS, HMGA2, MYC and MET.
MicroRNA loss	DNA methyltransferases	↓ for example p16, FHIT, and WWOX.
MicroRNA loss	Chromatin silencers	↓ of tumour suppressors.

Figure 3.3 *Consequences of aberrant miRNA expression.*

↑ denotes upregulation and ↓ denotes downregulation.

Adapted from Croce, CM (2009).

3.1.2.1 Tumour Suppressor miRNAs in Prostate Cancer

miR-15a/miR-16-1

MicroRNAs encoded by the miR-15/miR-16 cluster target a number of critical oncogenes including; BCL2, CCND1, MCL1 and WNT3A, thus within a normal setting their expression exerts a tumour suppressive effect (Cimmino *et al.,* 2005). However, the downregulation of these miRNAs as a result of a 13q14 deletion has been reported in many solid tumour types, including prostate carcinoma (Aqeilan *et al.,* 2010). The *in vivo* knockdown of these miRNAs has been shown to cause upregulation of WNT3A, a gene known to promote tumorigenic features including proliferation and invasion (Aqeilan *et al.,* 2010). Furthermore, their loss is known to result in hyperplasia associated with the subsequent upregulation of their oncogenic targets (Aqeilan *et al.,* 2010).

miR-101

Loss of miR-101 has been demonstrated in both clinically localised and disseminated prostate cancer, and results in the overexpression of EZH2, a polycomb family-associated histone methyltransferase. EZH2 (enhancer of zeste homolog 2) functions in the epigenetic

silencing of target genes and its overexpression as a result of miRNA dysregulation contributes to the survival and metastasis of malignant cells (Varambally *et al.,* 2008).

miR-200b

The miR-200 family is comprised of several critical tumour suppressor microRNAs, whose downregulation has been implicated in the progression of prostate cancer (Vrba *et al.,* 2010). miR-200c and miR-141, members of this family of miRNAs, are intimately involved in the maintenance of an epithelial phenotype. Dysregulated expression of these miRs has been demonstrated to induce epithelial-mesenchymal transition (EMT). As a result, abnormal expression of this miR family is associated with a more aggressive form of prostate cancer. Vrba *et al.,* (2010) have shown that aberrant DNA methylation of the miR-200c/miR-141 CpG island causes inappropriate silencing of these miRNAs and as a result induces a mesenchymal phenotype, indicating that epigenetic modifications may be responsible for the downregulation of the miR200 family in prostate cancer.

miR-146a

Loss of miR-146a has been implicated in the development of hormone-refractory prostate cancer. Lin *et al.,* (2008) found that miR-146a was robustly expressed in androgen-dependent prostate carcinoma cells and downregulated in androgen-independent cells. They demonstrated that constitutive overexpression of this miRNA resulted in increased expression of *ROCK1* and a concomitant reduction in proliferative capacity and metastatic ability. ROCK1 has previously been identified as a critical kinase in the transformation of hormone-refractory prostate cancer (Lin *et al.,* 2007). Thus, these findings indicate that miR-146a plays a tumour suppressive role in preventing ROCK-mediated malignant transformation in human prostate epithelial cells.

miR-330

Another critical tumour suppressor miRNA whose abnormal expression has been implicated in prostate cancer is miR-330. Overexpression of miR-330 in PC-3 cell lines has been demonstrated to reduce E2F-mediated Akt phosphorylation, thereby inducing apoptosis and abrogating cell viability (Lee *et al.,* 2009). miR-330 resides on chromosome 19q13 and has been shown to be specifically downregulated in both aggressive prostate cancer cell lines and clinical specimens, indicating that abnormal miR-330 expression may predispose to an aggressive prostate cancer subtype (Lee *et al.,* 2009).

miR-34a

The expression of miR-34a is induced in response to DNA damage in a p53-dependent manner, resulting in the downregulation of cell cycle control genes such as CDK4, cyclin D1, cyclin E2, E2F3 and BCL2, the overall effect of which is cell cycle arrest and apoptosis (Chang *et al.,* 2007). Loss of this tumour suppressor miRNA has previously been reported in prostate cancer specimens, particularly in patients with higher Gleason grade tumours (Kong *et al.,* 2013). Furthermore, miR-34a expression is absent in the androgen-independent PC-3 and DU145 prostate carcinoma cell lines. Ectopic expression of miR-34a in these cell lines has been shown to affect cell growth and inhibit the expression of SIRT1 (Fujita *et al.,* 2008). miR-34a inhibition of SIRT1 results in an increase in acetylated p53 and elevated expression of transcriptional targets of p53, such as p21 and PUMA (Yamakuchi *et al.,* 2008). As miR34a is a transcriptional target of p53, these findings indicate the existence of a positive feedback loop whereby miR-34a induces p53 activity through transcriptional silencing of SIRT1.

3.1.2.2 Oncogenic miRNAs in Prostate Cancer

miR-21

Abundance of the oncogenic microRNA miR-21 has been widely reported for many solid tumour types, including ovarian, thyroid, head and neck carcinomas and prostate cancer (Iorio *et al.,* 2007; Tetzlaff *et al.,* 2007; Tran *et al.,* 2007; Volinia *et al.,* 2006). MiR-21 has been shown to be overexpressed in both PC-3 and DU145 cell lines, and silencing of this miRNA impairs cell motility and invasion (Li *et al.,* 2009). Li *et al.,* (2009) also identified myristoylated alanine-rich protein kinase C substrate (MARCKS) as a target of miR-21. The effects of dysregulated miR-21 expression are postulated to be caused in part by aberrant MARCKS levels as this protein is heavily involved in varying cellular processes such as cell adhesion, motility and membrane trafficking (Arbuzova *et al.,* 2002). The majority of validated miR-21 targets are genes involved in the inhibition of cell migration and invasion including PTEN (Meng *et al.,* 2007) and TIMP3 (Gabriely *et al.,* 2008), which underscores the oncogenic identity of this microRNA.

miR-221/miR-222

miR-221 and miR-222 have emerged as two of the most consistently overexpressed microRNAs in human cancer. These closely related miRs have been shown to be aberrantly expressed in both haematopoietic and solid malignancies including chronic lymphocytic leukaemia, hepatocellular carcinoma, pancreatic, colon and prostate cancer (Calin *et al.,*

2005; Garzon *et al.,* 2008; Bloomston *et al.,* 2007; Schetter *et al.,* 2008; Ambs *et al.,* 2008). The forced overexpression of these miRNAs in LNCaP cells has been demonstrated to profoundly enhance not only their growth potential but also their tumorigenic and clonogenic capacities. Furthermore, inhibition of miR-221/222 in PC-3 cells results in impaired tumour-forming ability *in vivo* (Mercatelli *et al.,* 2008). Overexpression of these miRNAs is postulated to inhibit the expression of the tumour suppressors p27, p57, PTEN and TIMP3 resulting in an invasive malignant phenotype.

miR-125b

Dysregulated miR-125b expression has been implicated in the development of androgen-refractory prostate cancer (Shi *et al.,* 2007). Overexpression of miR-125b has been widely reported in clinical prostate cancer specimens. In addition, several studies have demonstrated elevated expression of miR-125b in high-grade, invasive tumours (Mitchell *et al.,* 2008). miR-125b is known to target a number of critical proapoptotic tumour suppressor genes including p53, Bak1 and PUMA (Shi *et al.,* 2007). miR-125b has also been implicated in the inhibition of the protein product of the ink4a/ARF locus, p14 in the prostate carcinoma cell lines LNCaP and 22Rv1 (Amir *et al.,* 2013). Along with Mdm2, $p14^{ARF}$ is a critical modulator of p53 activity and these results indicate that overexpression of miR-125b prevents the downregulation of Mdm2 by $p14^{ARF}$ which concomitantly affects the downstream effectors of p53. In support of this hypothesis, the inhibition of miR-125b has been demonstrated to increase the expression of $p14^{ARF}$ and reduce expression of Mdm2. These molecular alterations are sufficient to reduce cellular proliferation and induce apoptosis (Amir *et al.,* 2013). These findings suggest that miR-125b may represent a putative therapeutic target for the treatment of metastatic prostate cancer.

miR-20a

miR-20a resides in the miR-17-92 cluster, whose dysregulated expression has been observed in many types of human cancer. There is much evidence to suggest that members of this cluster function as both tumour-suppressor miRs and oncomiRs. An amplification event involving this region has been described in lymphoma and lung cancer, while loss of heterozygosity of the genes within this cluster has been observed in breast cancer (Mu *et al.,* 2009; Hayashita *et al.,* 2005; Hossain *et al.,* 2006). Qiang *et al.,* (2014) have described a significant overexpression of miR-20a in prostate cancer tissue when compared to benign tissue. Furthermore, this study observed that patients displaying increased levels of miR-20a possess higher Gleason grade tumours. In support of the role of miR-20a in prostate cancer progression, Qiang *et al.,* (2014) have demonstrated that miR-20a targets ABL2 (also known as Arg), a member of the ABL family of non-receptor tyrosine kinases. These proteins are

critical modulators of many diverse cellular functions including, proliferation, adhesion and migration (Greuber *et al.,* 2013). Loss of ABL2 increases prostate cancer cell invasiveness, indicating that miR-20a overexpression may play a critical role in prostate cancer progression.

There is an abundance of evidence to support the notion that miRNA alterations are involved in the initiation and progression of human malignancy. The same genomic abnormalities which were once believed to solely alter the expression and activity of protein-coding genes are now known to affect small regulatory members of the ncRNA class. In recent years, technological advances have facilitated the miRNA expression profiling of human tumours and these studies have been successful in uncovering miRNA expression signatures associated with disease staging, diagnosis, prognosis and treatment response. However, at present there remains no reliable predictive signature for prostate cancer accurate enough to replace morphology-based prognostics. As a result, the effective management of prostate cancer is hindered by the clinical burden of overtreatment and the inability to definitively identify inherently aggressive prostate neoplasms.

3.1.3 Experimental Hypothesis and Aims

miRNA dysregulation has been established as a significant pathogenic event in human cancer. In addition, there is much evidence to support the role of miRNAs as minimally invasive, robust biomarkers for detection not only in solid tumours but also in circulation. To this end, the hypothesis of this work was that there exists a specific miRNA expression signature, which characterises clinically aggressive prostate carcinoma. In an attempt to identify this signature we have constructed a biologically relevant miRNA panel for investigation in a cohort of putatively indolent versus aggressive formalin-fixed paraffin-embedded (FFPE) archival radical retropubic prostatectomy (RRP) specimens. This miRNA panel was constructed through a combinatorial meta-analysis of the literature and microarray experiments previously performed within the laboratory.

The primary aims of these investigations were;

- To construct a cohort comprised of archival FFPE RRP specimens sub-classified as indolent or aggressive prostate cancer based on post-operative pathology reports as defined by a modified version of Epstein's criteria for the identification of clinically insignificant disease (Epstein *et al.,* 1994).

- To perform a feasibility miRNA expression analysis by qRT-PCR on a training cohort (n=20) and to subsequently validate the results of this expression analysis on a

larger test cohort (n=30). The miRNA panel will eventually be interrogated on a much larger scale in the 150-patient prostate cancer research consortium cohort (as detailed in Chapter 4).

- To construct a tissue microarray of this archival patient cohort (n=50).

- To perform a bioinformatic analysis to identify a number of biologically relevant downstream targets of the miRNA panel for interrogation.

- To perform and interpret immunohistochemistry on the tissue microarray, examining the expression of the identified gene targets.

3.2 Results

3.2.1 Sample Collection and Classification

The patient cohorts for this study comprised of age-matched (± 5 years) archival treatment-naive formalin-fixed paraffin-embedded radical retropubic prostatectomy specimens excised at St. James's Hospital between 2007 and 2012. These samples were originally collected for the Prostate Cancer Research Consortium (PCRC) biobank. Ethical consent was granted from the St. James's Hospital ethics committee. Post-operative pathology reports were consulted to identify the clinicopathological parameters for classification into clinically indolent and aggressive subgroups. The modified criteria employed for the identification of insignificant disease were derived from Epstein's criteria for disease classification in biopsy material (Epstein *et al.*, 1994). For putatively indolent disease we identified patients with <0.5 cm^3 tumour volume (where possible), Gleason score ≤ 6 (no Gleason pattern 4 present), and stage T2 disease (disease must be confined within the prostatic capsule). Aggressive disease was defined as >0.5 cm^3 tumour volume, Gleason score ≥ 7 and ≥ stage T2c disease (where possible). The clinical characteristics of the final patient samples used in both cohorts are listed in Table 3.1 and Table 3.2.

	All Patients (n = 20)	Indolent (n = 10)	Aggressive (n = 10)	*P*
Age (y)				
Mean (Median)	63.4(60.5)	59.8(59)	61.4(61)	*0.064*
Range	50-69	50-67	54-69	
Pre-PSA (ng/ml)				
Mean (Median)	7.39(7.1)	6.605(6.55)	8.18(8)	*0.122*
Range	2.13-13	2.13-12.2	5.8-13	
Prostatectomy Stage (%)				
OC	12(60)	10(100)	2(20)	*0.148*
NOC	8(40)	0 (0)	8(80)	
Gleason Score (%)				
≤6	10(50)	10(100)	0 (0)	*0.185*
≥7	10(50)	0 (0)	10(100)	

Table 3.1 *Clinicopathological characteristics of the initial feasibility cohort.*

OC represents organ confined cases and NOC represents non-organ confined cases.

	All Patients (n = 30)	Indolent (n = 15)	Aggressive (n = 15)	*P*
Age (y)				
Mean (Median)	59.3(60)	60.2(60)	58.5(60)	*0.082*
Range	45-68	50-68	45-66	
Pre-PSA (ng/ml)				
Mean (Median)	6.48(5.6)	5.43(4.8)	8.12(6.5)	*0.154*
Range	1.6-28	2.13-10	1.6-28	
Prostatectomy Stage (%)				
OC	27(90)	15(100)	12(80)	*0.198*
NOC	3(10)	0 (0)	3(20)	
Gleason Score (%)				
≤6	15(50)	15(100)	0 (0)	*0.117*
≥7	15(50)	0 (0)	15(100)	

Table 3.2 *Clinicopathological characteristics of the validation cohort.*

OC represents organ confined cases while NOC represents non-organ confined cases.

3.2.2 Validation of Endogenous Control Genes for miRNA Expression Analysis

In order to choose a stable endogenous control for this study, a literature search was performed to identify the most commonly used endogenous control genes in miRNA expression analyses of prostate cancer specimens. RNU24 and RNU44 (small nucleolar RNAs) were consistently identified as robust housekeeper genes in prostate cancer (Carlsson *et al.*, 2010). The expression of these small RNAs was examined in six formalin-fixed paraffin-embedded samples comprising; one 'high-grade' prostate carcinoma (core, Gleason pattern 4), one' low-grade' prostate carcinoma (core, Gleason pattern 3), one benign sample (core), benign macrodissected tissue (from whole section on slide), and two whole sections both of which contained areas of high-grade prostatic intraepithelial neoplasia (HG-PIN). The average of the raw cycle threshold (C_T) values across the samples was compared to determine the variability of the genes. Let-7a has previously been reported as dysregulated in prostate cancer and was included as a comparison (Dong *et al.*, 2010; Wang *et al.*, 2013). RNU24 and RNU44 displayed similar cycle threshold values across the samples and very little variability was observed between the two (Figure 3.4). As expected the expression of let-7a was found to be highly variable across the samples. RNU24 was selected as the appropriate endogenous control gene for this study as it was found to display consistent C_T values across the varying sample types and its utility as a robust housekeeper gene for miRNA expression analysis in prostate cancer has been experimentally endorsed (Carlsson *et al.*, 2010).

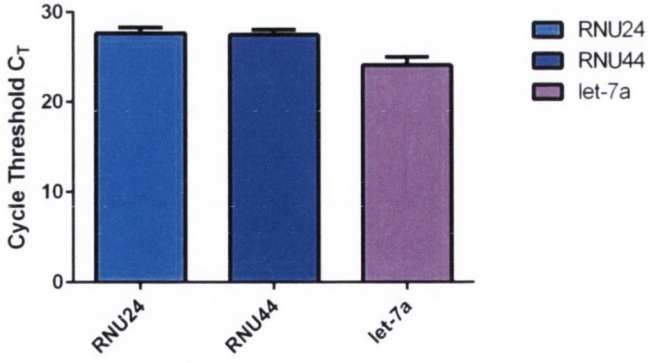

Figure 3.4 *Cycle threshold comparison for potential endogenous control genes across varying samples (n=6).*

Very little difference was observed in the mean cycle threshold values between RNU24 and RNU44. Let-7a was included as a comparator to demonstrate its variability in prostate cancer. Data is graphed as mean and standard error of the mean.

3.2.3 miRNA Expression Analysis in Feasibility Cohort

Quantitative RT-PCR was employed to examine the expression of this panel of miRNAs in the feasibility patient cohort comprising of putatively aggressive (n=10, Table 3.1) and indolent (n=10, Table 3.1) radical prostatectomy specimens. Four miRNAs were statistically significantly differentially expressed in the aggressive versus indolent patient groups; miR-141, miR-146a, miR-200b and miR-20a. These four miRNAs displayed a significant upregulation in the aggressive patient group. No significant differential expression was observed for the remaining miRNAs in the panel (Figure 3.1).

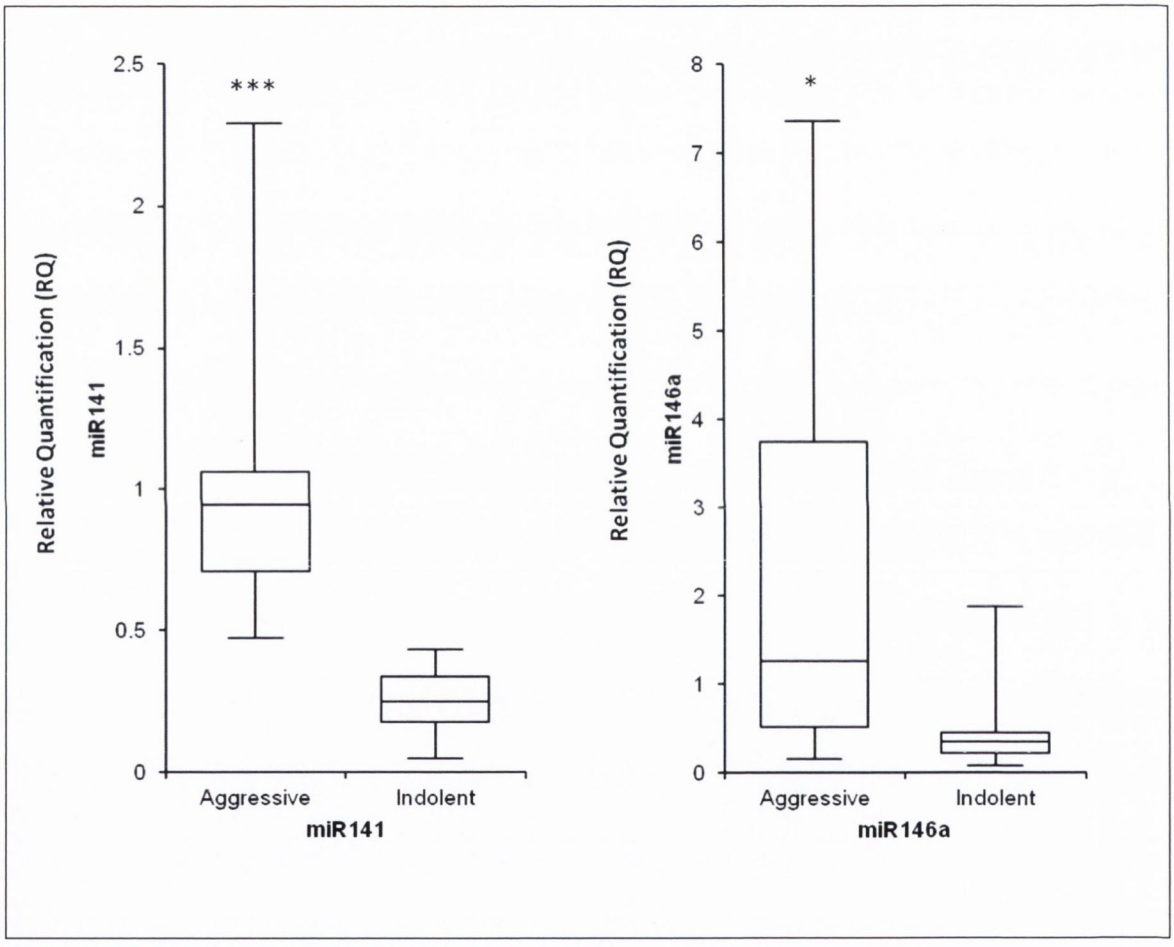

Figure 3.5 *Boxplots of relative quantification (RQ) of change in expression of miR-141 and miR-146a.*

miR-141 and miR-146a were statistically significantly upregulated in the aggressive patient cohort. Statistical significance: Mann-Whitney U Test, +/- 1.5-fold change, $p < 0.05$. *** denotes $p < 0.001$.

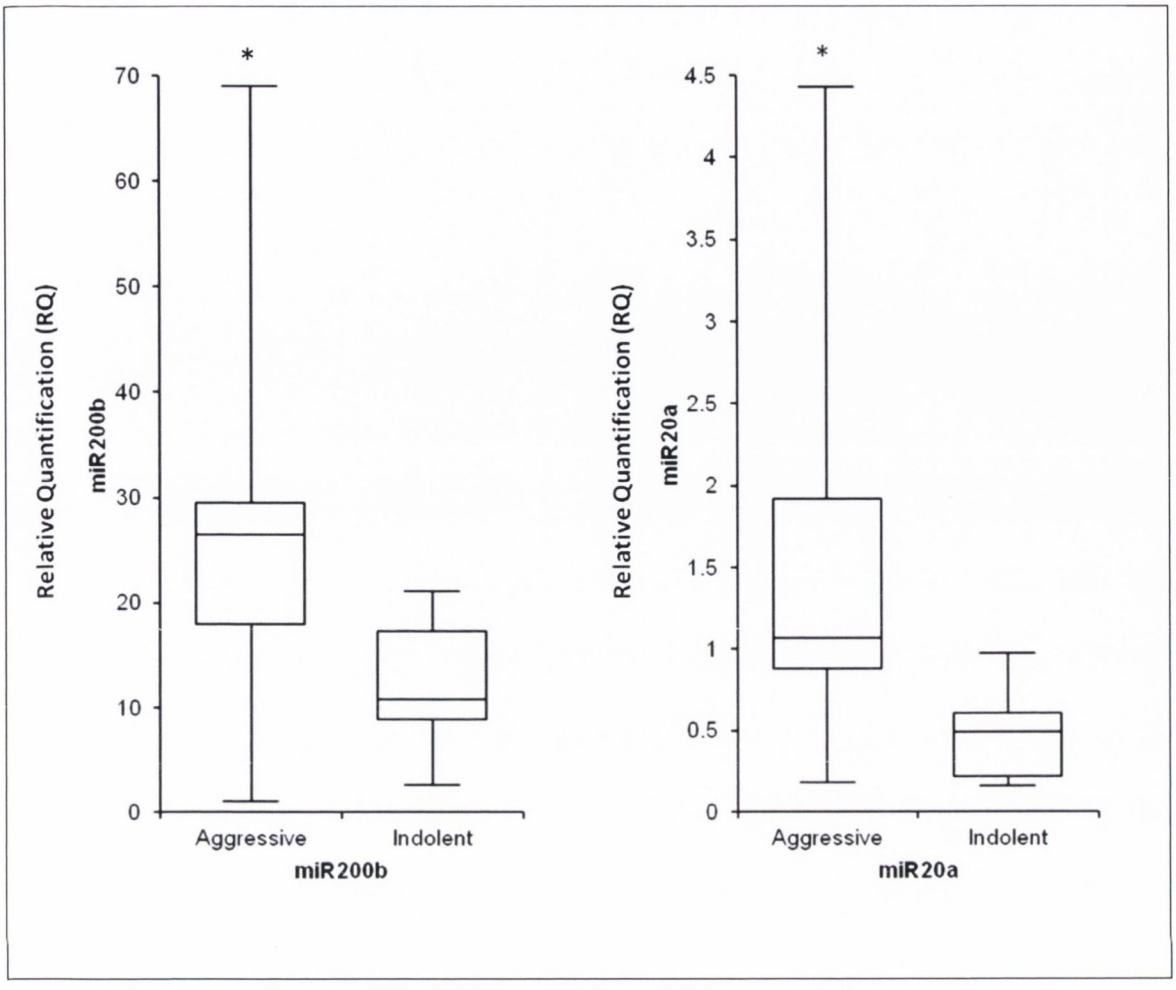

Figure 3.6 *Boxplots of relative quantification (RQ) of change in expression of miR-200b and miR-20a.*

miR-200b and miR-20a were statistically significantly upregulated in the aggressive patient sample set. Statistical significance: Mann-Whitney U Test, +/- 1.5-fold change, $p < 0.05$.

3.2.4 miRNA Expression Analysis in Validation Cohort

To perform a validation of the differential miRNA expression profiles observed, expression of the same panel of miRNAs was examined in an extended test cohort comprising n=15 aggressive (Table 3.2) and n=15 indolent (Table 3.2) FFPE clinical specimens. miR-125b, miR-20a, miR-146a and miR-200b were found to be statistically significantly differentially expressed, however contradictory to the findings of the initial study, they were found to be significantly upregulated in the indolent patient set (Figure 3.7, Figure 3.8). No significant difference in the expression pattern of miR-141 was observed between the aggressive and indolent patient groups in the validation cohort despite this miRNA being identified as significantly differentially expressed in the initial study. No significant differential expression was observed for the remaining miRNAs in the panel (Figure 3.1). Review of experimental conditions showed no technical errors had been made.

In order to address this discrepancy in results, the patient cohorts were rearranged and data was reanalysed as organ-confined versus non organ-confined disease using the same statistical methods. The results of this analysis demonstrated that no miRNA within the cohort was statistically significantly differentially expressed.

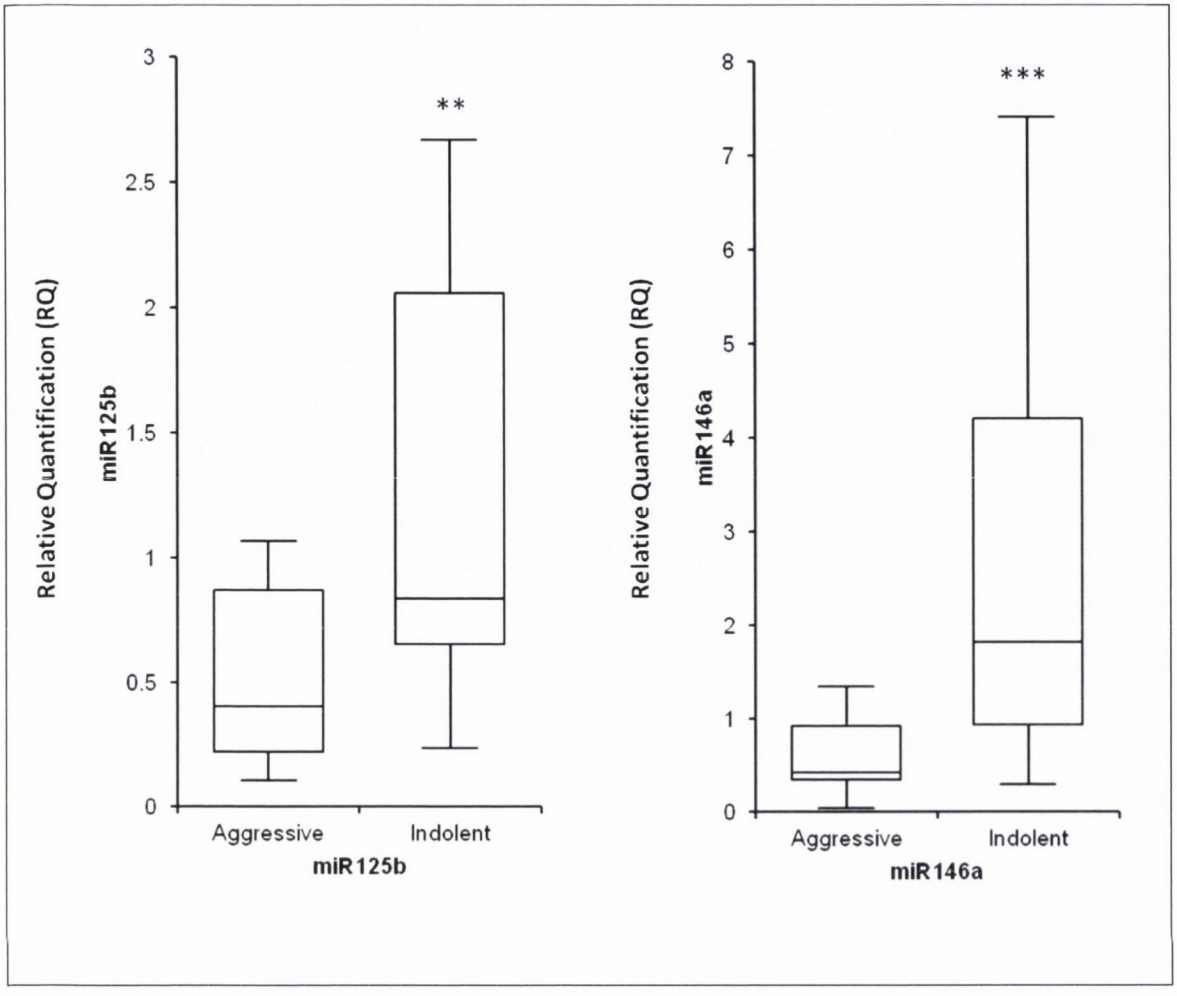

Figure 3.7 *Boxplots of relative quantification (RQ) of change in expression of miR-125b and miR-146a.*

miR-125b and miR-146a were found to be statistically significantly upregulated in the indolent patient group. Statistical significance: Mann-Whitney U Test, +/- 1.5-fold change, $p < 0.05$. ** denotes $p < 0.01$ *** denotes $p < 0.001$.

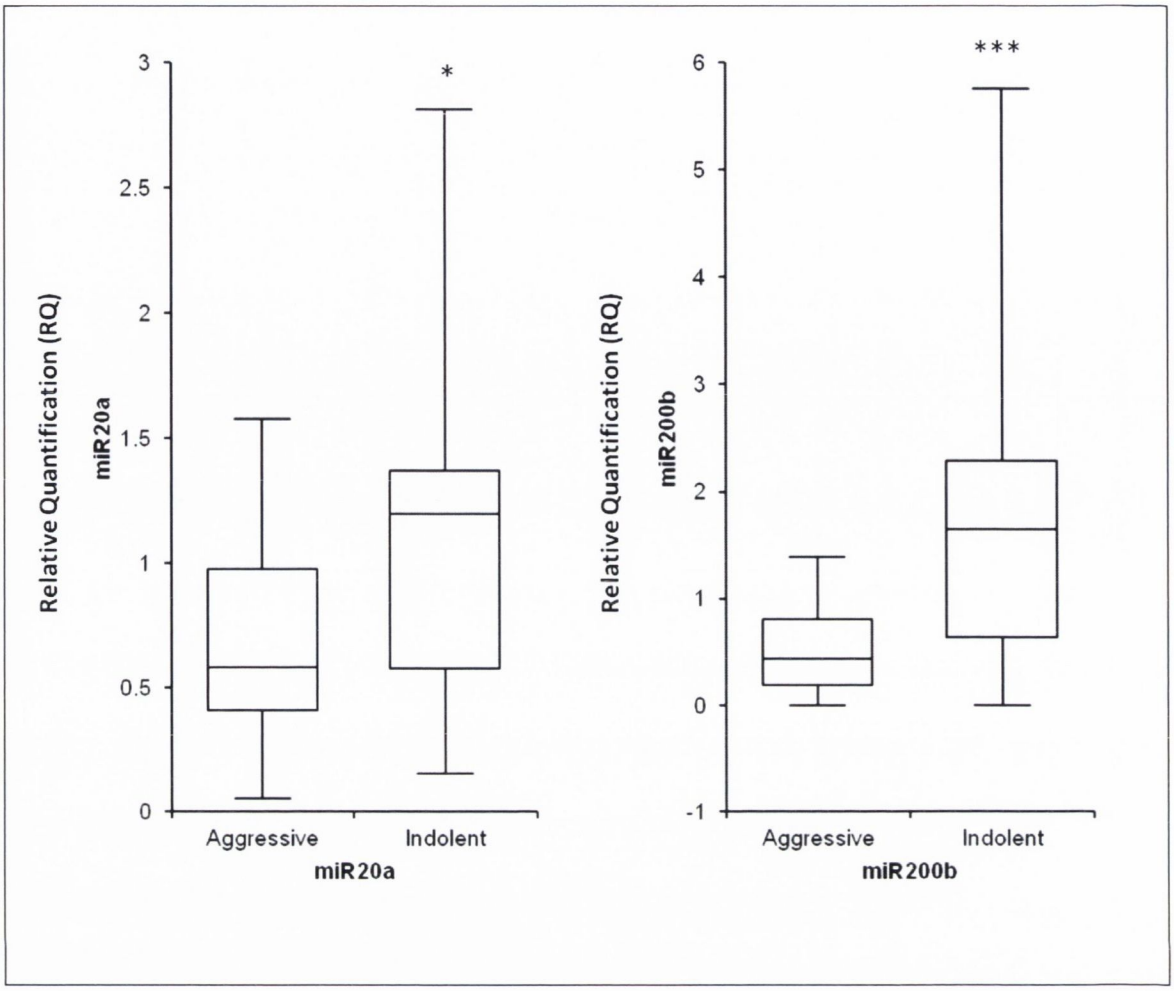

Figure 3.8 *Boxplots of relative quantification (RQ) of change in expression of miR-20a and miR-200b.*

miR-20a and miR-200b were found to be significantly upregulated in the aggressive patient group in our initial study, however examination of their expression profiles in a validation cohort identified a significant upregulation of these miRNAs in the indolent patient group. Statistical significance: Mann-Whitney U Test, +/- 1.5-fold change, $p < 0.05$. *** denotes $p < 0.001$

3.2.5 Bioinformatic Investigation of Downstream Targets

A bioinformatic computational prediction of miRNA targets was performed using the target prediction program 'miRWALK', to identify experimentally validated gene targets of the miRNA panel for analysis in a tissue microarray. miRWALK is a comprehensive database of both predicted and validated targets of miRNAs for human, mouse and rat genomes. The validated target module of this database allows the input of a miRNA candidate and provides target gene names, Entrez IDs and individual hyperlinks to the PubMed database. Each member of the miRNA panel was probed in this database. A pathways analysis was then performed by cross referencing the Entrez IDs of the validated targets of each miRNA using the functional annotation tool DAVID (**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery). The resultant KEGG pathway (**K**yoto **E**ncyclopaedia of **G**enes and **G**enomes) provided a graphical schematic of the molecular target dataset of each miRNA, which allowed the identification of the most biologically relevant downstream genes. miR-200b, miR-141, miR-125b and miR-20a were all found to directly target genes within the same pathway (Figure 3.9, Figure 3.10, Figure 3.11, Figure 3.12). A key regulator of G_1 phase progression in the cell cycle, cyclin D1 (CCND1), was found to be a common target of each of these four miRNAs. A subsequent literature search of the data published on cyclin D1 revealed there is much evidence linking overexpression of this protein to tumour progression in multiple cancer types (Lamb *et al.,* 2003). In addition, aberrant cyclin D1 expression has previously been associated with the development of androgen-independent prostate cancer (Drobnjak *et al.,* 2000). Furthermore, a number of papers were identified, describing a statistical association between cyclin D1 overexpression and high Ki-67 proliferative index (Drobnjak *et al.,* 2000; Aaltomaa *et al.,* 2006). Aaltomaa *et al.,* (2006) observed a direct relationship between coordinated high expression of these proteins and malignant histological features, indicating a potential prognostic role for these markers. Thus, both cyclin D1 and Ki-67 were chosen as candidates for investigation in the patient cohort. Target prediction analysis also revealed that a miRNA candidate within the panel, miR-221, directly targeted ERG (ETS-related gene). Due to the widely reported role of this gene in prostate cancer as a result of the recurrent chromosomal translocation involving the TMPRSS2 gene, ERG was also chosen for further analysis in the tissue microarray (Tomlins *et al.,* 2005).

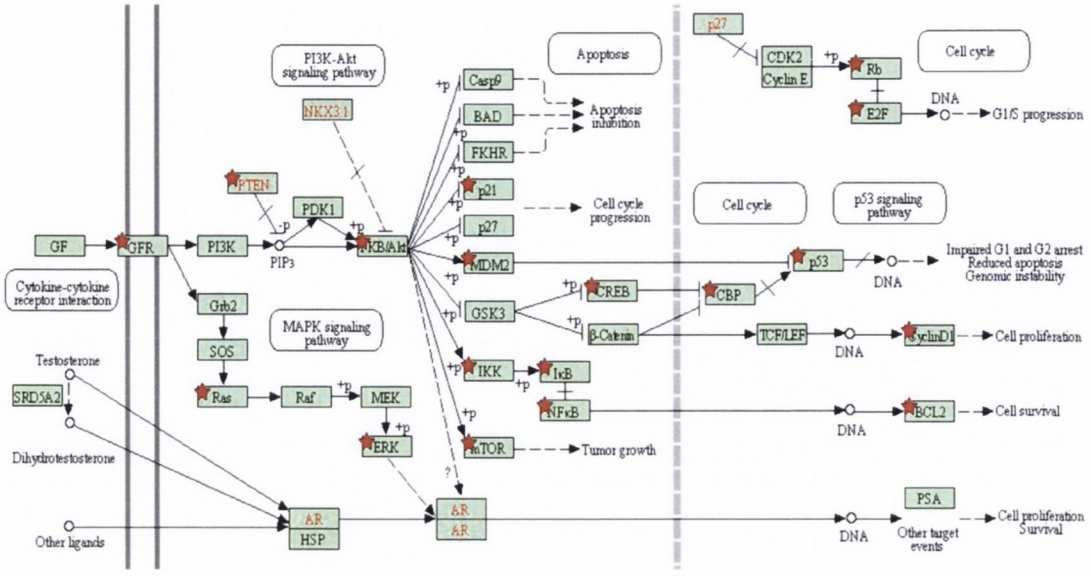

Figure 3.9 *KEGG pathway for miR-125b compiled by functional annotation analysis using DAVID.*

Red stars denote mRNA targets of miR-125b. This network provides a visual representation of the molecular pathways regulated by miR-125b expression. miR-125b, miR-200b, miR-141 and miR-20a were all found to target genes within the same commonly dysregulated pathway in human cancer.

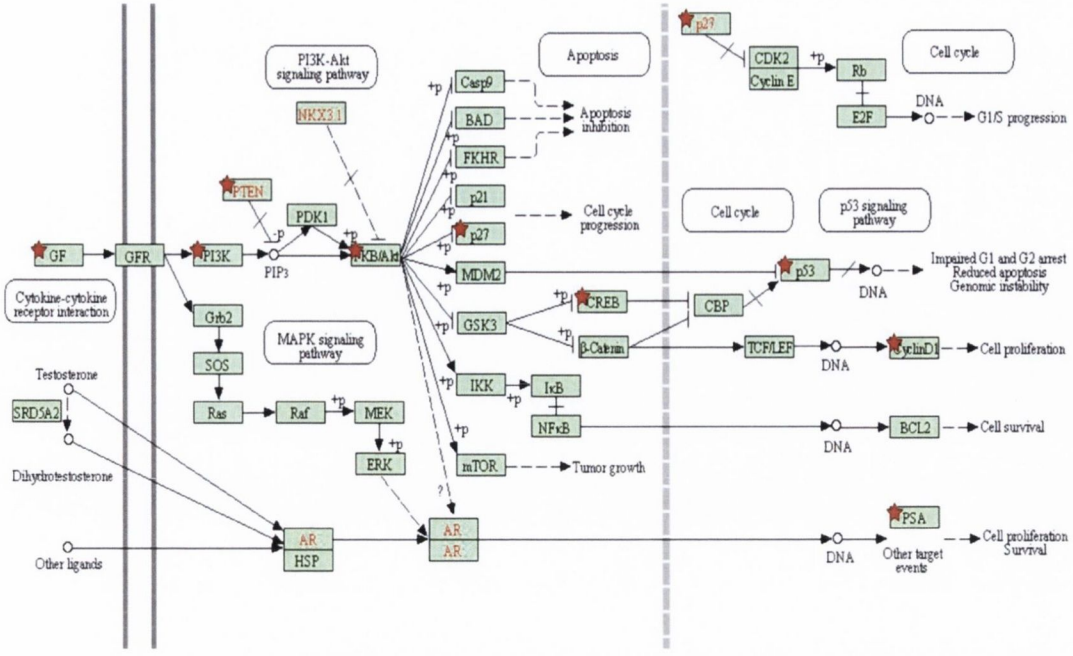

Figure 3.10 *KEGG pathway for miR-141 compiled by functional annotation analysis using DAVID.*

Red stars denote mRNA targets of miR-141 and where they lie in relation to multiple molecular pathways.

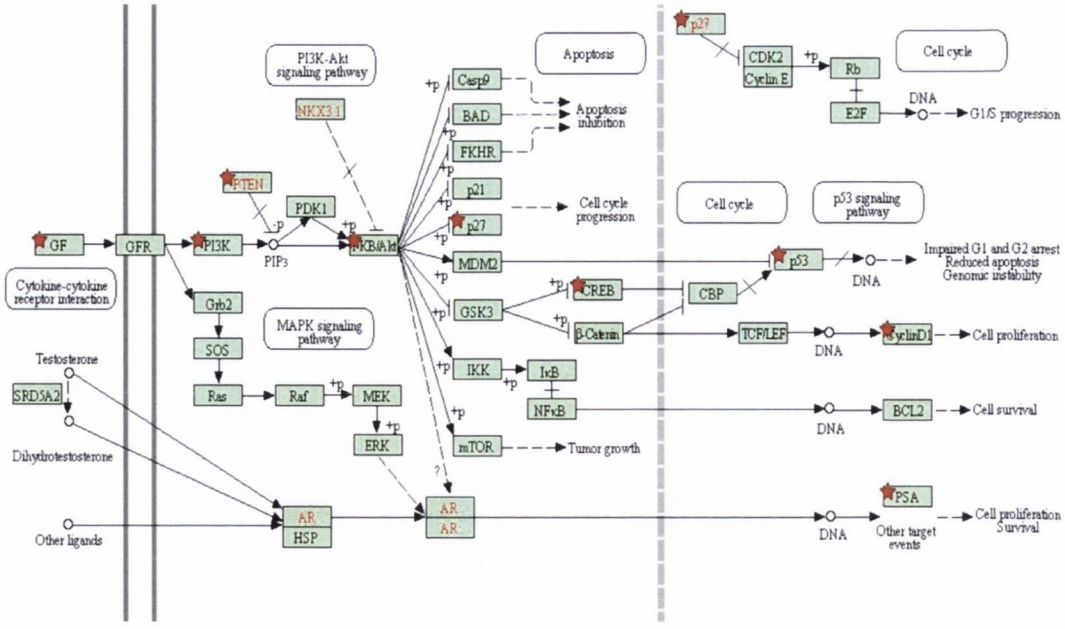

Figure 3.11 *KEGG pathway for miR-200b compiled by functional annotation analysis using DAVID.*

Red stars denote the mRNA targets of miR-200b.

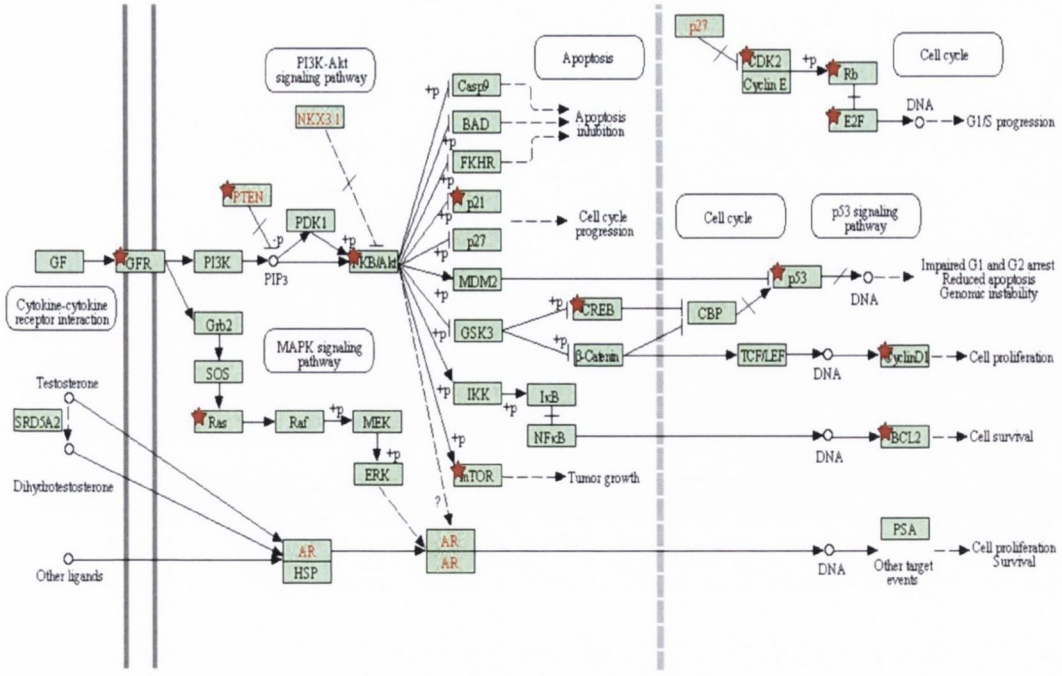

Figure 3.12 *KEGG pathway for miR-20a compiled by functional annotation analysis using DAVID.*

Red stars denote the mRNA targets of miR-20a.

miRNA	Gene Target
miR-200b	CCND1, GF, PTEN, PI3K, Akt, p27, CREB, p53, p27, PSA
miR-141	CCND1, GF, PTEN, PI3K, Akt, p27, CREB, p53, p27, PSA
miR-125b	CCND1, GFR, PTEN, Ras, ERK, mTOR, IKK, CREB, MDM2, CBP, p53, BCL2, RB, E2F
miR-20a	CCND1, GFR, PTEN, Ras, Akt, mTOR, CREB, CDK2, p53, RB, E2F, BCL2

Table 3.3 *Summary of gene targets for each miRNA common to the pathway mentioned.*

miR-200b, miR-141, miR-125b and miR-20a were all found to target cyclin D1.

3.2.6 Immunohistochemical Assessment of Ki-67, Cyclin D1 and ERG

Prior to construction of the tissue microarray, 5μm sections were taken from each constituent block and stained with haematoxylin and eosin (H&E) for marking of morphologically representative areas of the tumour to ensure these areas had not diminished during extraction for the miRNA component of this study. Three cases were found to contain insufficient tumour material. Tissue cylinders with a diameter of 0.6 mm were removed from three targeted areas (of the dominant tumour nodule) of each constituent donor block and deposited into a recipient TMA block. The final TMA block consisted of 65 cores; 47 prostatectomy tissues and 18 built-in no tumour controls. Five additional human spleen tissue cores were included in order to correctly orientate the block. The whole tissue sections and stained TMA slides were scored by the study pathologist (Figure 3.13, Figure 3.14, Figure 3.15). The primary antibodies and antigen retrievals used in this study are listed in Chapter 2. Known positive tissues were used as positive controls for each marker, while the primary antibody was omitted for negative controls.

Varying immuno-reaction indices were utilised to quantify each marker analysed. Cyclin D1 was represented as the percentage positive nuclei within the tumour of all three sections per case. For statistical analysis cyclin D1 staining was divided into tertiles; 0-40%, 40-70%, >70% (Table 3.4) (Histograms of raw and averaged protein expression values can be found in Appendix 1).

Ki-67 was quantified by the 'proliferative index'; the mean fraction of positive nuclei was estimated. For analysis, Ki-67 was grouped into two categories; 0-2% and >2%. These cutoffs were chosen based on previous studies (Aaltomaa *et al.,* 2006) (Table 3.4).

The intensity of ERG was scored as 0 (negative), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining) and these scores were used to calculate Allred scores in order to dichotomise ERG staining into presence/absence (Table 3.4). Allred scores are based on the percentage of cells, which stain positive for a chosen marker. Cores are first assigned a proportion score, which defines the percentage of cells staining positive. Cores are then assigned an intensity score (as described above). The Allred score is then calculated by adding the proportion score and the intensity score (Allred *et al.,* 1993).

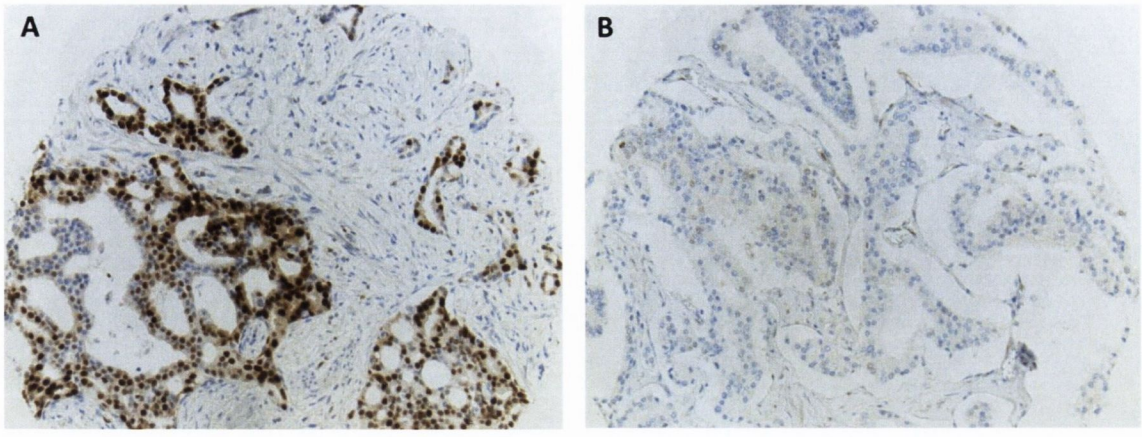

Figure 3.13 *Representative CCND1 (cyclin D1) staining in selected 0.6mm prostate tumour cores in the tissue microarray.*

(A) Strongly positive staining for the expression of cyclin D1 (20X). (B) Negative expression of cyclin D1 (20X).

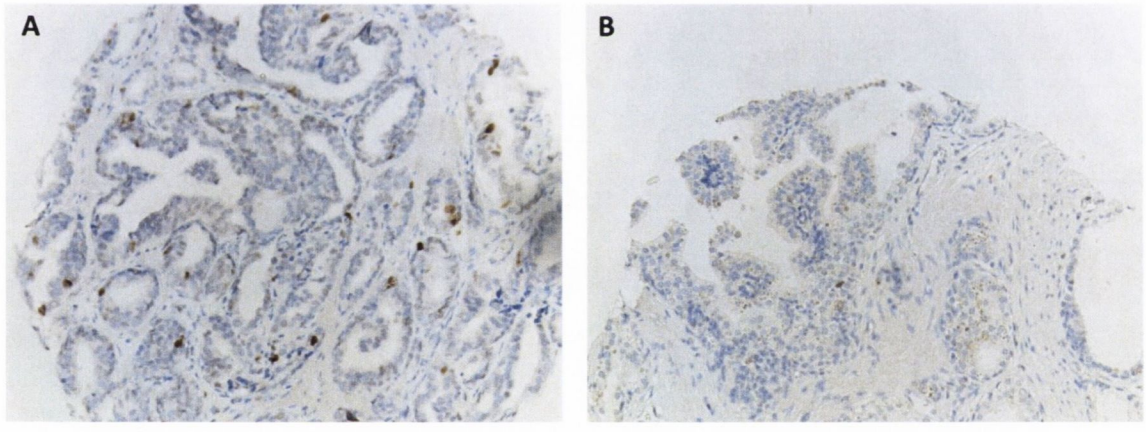

Figure 3.14 *Representative Ki-67 staining in selected 0.6mm prostate tumour cores in the tissue microarray.*

(A) Example of nuclear positive staining for Ki-67 expression (20X). (B) Weakly positive/low expression of Ki-67 (20X).

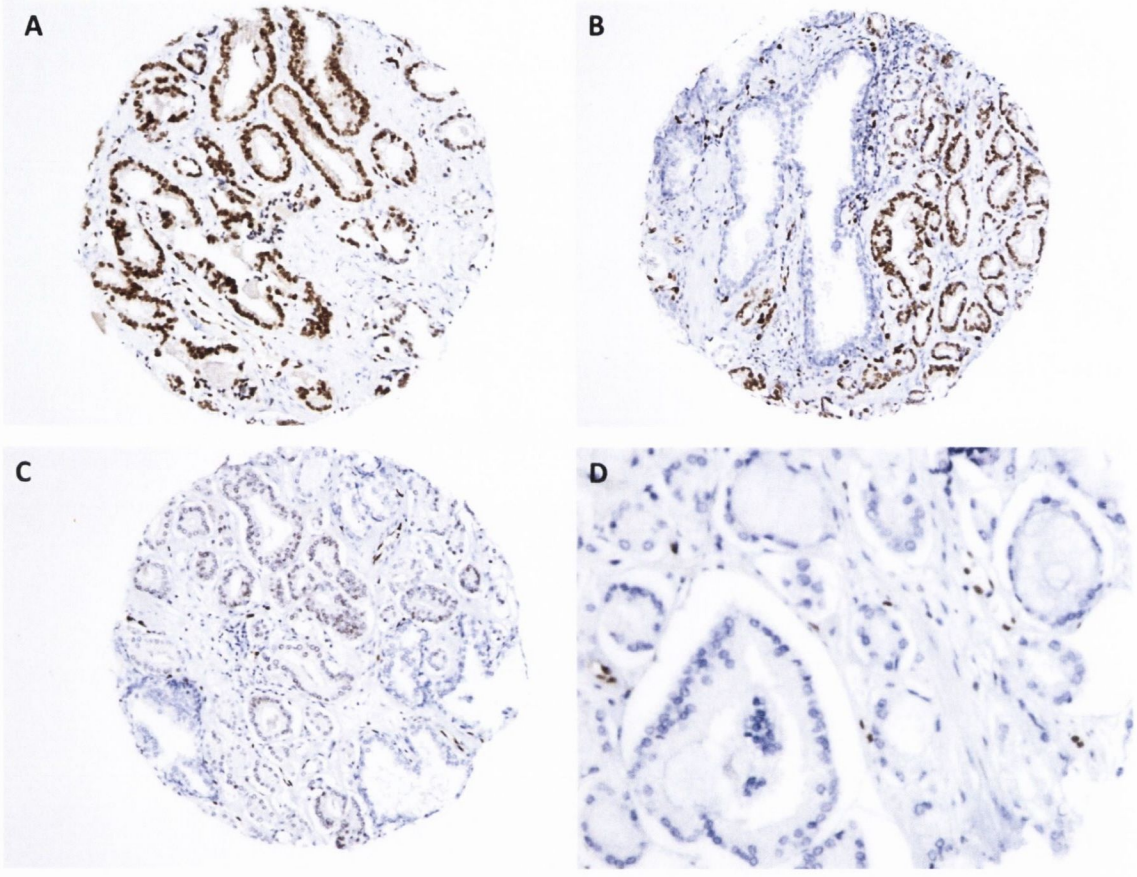

Figure 3.15 *Representative ERG (ETS-related gene) staining in selected 0.6mm prostate tumour cores in the tissue microarray.*

(A) Strongly positive staining for ERG (10X). (B) Strong positive staining for ERG (10X). (C) Weakly positive staining for ERG (10X). (D) Negative staining for ERG with a positive endothelial control (40X).

Variable	Frequency (%)
(% positively stained)	
Ki-67	
0-2%	27(57.45)
>2%	20(42.55)
CCND1	
0-40%	10(21.28)
40-70%	12(25.53)
>70%	25(53.19)
ERG	
Positive	24(51.06)
Negative	23(48.94)

Table 3.4 *Results of Ki-67, Cyclin D1 (CCND1) and ERG immunohistochemistry.*

Frequency refers to the total number of patients, which fall within each category while the corresponding percentage of the total cohort is indicated as (%).

3.2.7 Ki-67 Proliferative Index Statistically Significantly Associated with Gleason Score and Pathological Stage

The expression phenotypes of Ki-67 were dichotomised as 0-2% (low) and >2% (high). Low Ki-67 expression was observed in 27 of 47 radical prostatectomy cases (57.45%), while high Ki-67 expression was observed in 20 of 47 radical prostatectomy cases (42.55%). A significant association was observed between high Ki-67 proliferative index and Gleason score ($p = 0.009$) and pathological stage ($p = 0.0203$). Furthermore, an association was observed between high Ki-67 index and tissue type (putatively indolent vs. aggressive) ($p = 0.0254$). No association was observed between Ki-67 index and preoperative PSA ($p = 0.22$).

	Total no. of patients (%)	Ki-67 (0-2%)	Ki-67(>2%)	*p*
Pre-operative PSA (ng/ml)				
0-3.9	5(12.82)	2(5.12)	3(7.69)	0.22
4-9.9	29(74.35	19(48.71)	3(7.69)	
>9.9	5(12.82)	1(2.56)	10(25.64)	
Gleason Score				
≤6	22(46.81)	17(36.17)	10(21.28)	0.0099
≥7	25(53.2)	5(10.64)	15(31.91)	
Pathological Stage				
≤pT2c	33(73.3)	22(48.8)	11(24.4)	0.0203
≥pT3a	12(26.6)	3(6.6)	9(20)	

Table 3.5 *Clinicopathological parameters in relation to Ki-67 immunoreactivity.*

High expression of Ki-67 is associated with higher Gleason scores while low Ki-67 expression is associated with a pathological stage of T2c or less.

Tissue Type	Ki-67 Expression		*p*
	0-2 (%)	>2(%)	0.0254
Indolent	17(36.17)	6(12.77)	
Aggressive	10(21.28)	14(29.79)	

Table 3.6 *Tissue type in relation to Ki-67 immunoreactivity.*

Figures are represented as total number of patients and percentage of the cohort.

3.2.8 Statistical Association Exists Between CCND1 Overexpression and High Ki-67 Proliferative Index

High cyclin D1 expression (>70% tumour cells displaying nuclear immunostaining) was observed in 25 of 47 radical prostatectomy cases (53.19%) (Table 3.4). The cyclin D1 positive phenotype (≥ 40% nuclear immunostaining) was observed in 37 of 47 radical prostatectomy cases (78.72%). No association was observed between high cyclin D1 expression and clinical variables of; Gleason score ($p = 0.9471$), pathological stage ($p = 0.2034$), preoperative PSA ($p = 0.4523$) or tissue type (putatively indolent vs. aggressive) ($p = 0.4247$). However, a strong association was observed between cyclin D1 overexpression and high Ki-67 proliferation index ($p = 0.0051$) (Table 3.7). High proliferative index (>2% of tumour cells displaying nuclear Ki-67 immunoreactivities) was detected in 20 of 47 radical prostatectomy cases (42.55%).

	CCND1			*p*
Ki-67	0-40%	40-70%	>70%	0.0051
0-2%	9(19.15)	9(19.15)	9(19.15)	
>2%	1(2.13)	3(6.38)	16(34.04)	

Table 3.7 *Ki-67 proliferation index in relation to cyclin D1 immunoreactivity.*

Figures are represented as total number of patients and percentage of the cohort.

3.2.9 Expression of CCND1 and ERG Positivity are Significantly Interrelated

ERG expression was quantified as positive/negative using Allred scoring (Allred *et al.*, 1998). 23 of 47 radical prostatectomy cases (48.94%) were identified as negative for ERG expression, while 24 of 47 radical prostatectomy cases (51.06%) were positive for ERG expression. No association was observed between the ERG positive phenotype and Gleason score (p = 0.8911), pathological stage (p = 0.3098), preoperative PSA (p = 0.8221) or tissue type (p = 0.8815). However, a significant association was identified between ERG positivity and elevated cyclin D1 expression (p = 0.0013) (Table 3.8). In addition, this interrelationship was found to be significantly associated with patient age at diagnosis (p = 0.0354).

The relationship between ERG positivity and Ki-67 proliferative index was also investigated, however no statistically significant association was identified (Appendix 1).

	CCND1			*p*
ERG	0-40%	40-70%	>70%	0.0013
Positive	10(21.28)	4(8.51)	9(19.15)	
Negative	0(0)	8(17.02)	16(34.04)	

Table 3.8 *ERG positivity in relation to cyclin D1 immunoreactivity.*

Figures are represented as total number of patients and percentage of the cohort.

3.3 Discussion

3.3.1 Validation Failed to Confirm Results of the Pilot Study

The effective management of prostate cancer is impeded by the inability to accurately predict disease progression, particularly amongst those patients with intermediate disease. Preoperative PSA, Gleason score and clinical stage currently form the basis upon which high-risk patients are identified (Sboner *et al.,* 2010). However, these clinicopathological parameters fail to accurately predict the prognosis of patients with clinically localised prostate tumours. Thus, the need to identify patients with intrinsically aggressive prostate cancer remains an ongoing challenge. An overarching aim of this thesis was to identify and explore the molecular mechanisms which govern the clinical nature of prostate cancer and in so doing identify a signature predicting a lethal or indolent disease course.

The failure of the validation expression analysis to confirm initial findings is likely the result of a number of study limitations. Firstly, there is a fundamental and potentially crucial difference between the training and the test cohorts. The 'aggressive' group of the training cohort comprised primarily of non-organ confined disease, while the 'aggressive' group of the test cohort comprised predominantly of organ-confined disease. This discrepancy in cohort design is likely contributing to the lack of concordance between studies; however this was unavoidable due to the unavailability of suitable clinical specimens. Secondly, the study sample size (n=50 in total) was relatively small; an issue which has confounded previous prostate cancer expression profiling studies. A study by Wei *et al.,* (2004) has highlighted the importance of sample size for detecting differentially expressed genes as a small number of biological replicates is believed to result in low statistical power and subsequent high false positive rates. However, while increasing sample size concomitantly increases statistical power, as previously mentioned, this is often not possible as suitable clinical samples are simply not available. Increasing sample size also raises concern regarding efficient use of invaluable resources, both of which are tangible challenges associated with the use of human clinical specimens.

Wei *et al.,* (2004) hypothesise that the determination of suitable sample size is dependent upon the degree of variability present within the population, which underscores the final major limitation of this study; the presence of considerable disease heterogeneity. A number of critical studies have reported the presence of multiple geographically and clonally distinct tumour nodules within the prostate gland which highlights how sampling of the 'correct' tumour nodule is of paramount importance (Barry *et al.,* 2007; Mehra *et al.,* 2007; Clark *et al.,* 2008). The inability to identify the molecularly dominant tumour lesion may in part explain the heterogeneity in patient outcome following initial diagnosis. While the presence of

putative intra-tumour heterogeneity may account for sampling errors, the existence of a high degree of heterogeneity between aggressive prostate cancer cases is most likely a crucial factor in the inability to detect a clear 'aggressive signal'. A potential explanation for this apparent clinical variability is the steady development of an aggressive signature over time as a result of the accrual of multiple distinct molecular anomalies, which may not be present at the time of diagnosis or following initial treatment (Sboner *et al.,* 2010). The equivocal results from this study prevent the extrapolation of any hard conclusions; however they do provide a cautionary reminder of the many limitations, which must be overcome in order to perform an accurate expression profiling study of putatively aggressive prostate cancer.

3.3.2 Ki-67 Proliferation Index is Associated with Clinical Characteristics

Ki-67 is a nuclear antigen present during all active phases of the cell cycle (G_1, S, G_2, and mitosis). Immunohistochemical quantification of the Ki-67 proliferative index provides a determination of the growth faction of a cellular population (Gerdes, 1990). Evidence has established a predictive role for Ki-67 measurement in determining disease outcome following radical prostatectomy (Bettencourt *et al.,* 1996). Furthermore, the prognostic potential of the Ki-67 proliferative measurement has also been established for prostate cancer-specific survival in patients monitored by watchful waiting (Borre *et al.,* 1998). A study by Jhavar *et al.,* (2009) sought to further investigate the prognostic utility of Ki-67 in prostate cancer patients managed through active surveillance. The patients in this study were deemed to have low- or intermediate-risk disease and most possessed ≤20% tumour involvement in any single diagnostic tissue biopsy core. The identification of a significant association between Ki-67 proliferative index and time to radical treatment in this patient cohort suggests that Ki-67 may represent a potential biomarker of prostate cancer behaviour. Our findings identified a significant association between clinical characteristics (Gleason score and pT classification) and high Ki-67 proliferative index. Therefore, Ki-67 labelling index was capable of distinguishing putatively indolent from aggressive cases based upon our division criteria. Unfortunately due to the immaturity of this cohort, it is impossible to determine whether Ki-67 expression alone is a strong predictor of cancer-specific outcome. However, our data does suggest that the quantification of Ki-67 may improve the predictive ability of currently used algorithms.

The findings of our study closely parallel those of many previous studies which have reported a strong association between abnormal Ki-67 expression and higher Gleason scores, more aggressive cancer phenotypes and higher rates of recurrence (Fisher *et al.,* 2013). Despite a large body of literature supporting Ki-67 as a predictive molecular marker in prostate cancer, it is prudent to question why this marker is not more prominently utilised

within a clinical setting. This is most likely a result of analytical issues which affect its accurate measurement. When one considers the many studies, which have demonstrated the prognostic value of Ki-67, it becomes clear that almost all of these studies are retrospective and include heterogeneous groups of patients who were treated and monitored using disparate methods. In addition, there is little consensus regarding the immunohistochemical assessment of Ki-67, particularly in terms of cutoffs to designate 'positive' or 'negative' and 'high' or 'low'. Infact, predictions of disease progression following primary treatment have been based upon a wide range from 2.4% to 26% (Fisher *et al.,* 2013). In addition, an interesting study examining Ki-67 assessment in breast cancer has claimed that there is anecdotal evidence to suggest that Ki-67 scores are generally lower on TMAs in comparison to whole sections, indicating that the use of TMAs may be inappropriate to establish cutoffs for clinical application on various other sample types (Dowsett *et al.,* 2011). While there are no published systematic comparisons of Ki-67 staining on TMAs versus whole sections it is worthy to note that such variability can exist.

There is no doubt that the Ki-67 measure of proliferation is important in clinical practice. As our study has demonstrated, Ki-67 is strongly associated with current histopathological parameters and as a result is mildly informative in terms of disease behaviour. The clinical feasibility of the Ki-67 proliferative index as a prognostic indicator may become more apparent were it to be incorporated into a multiparameter panel of biomarkers. However, it is unlikely that the widely reported potential of Ki-67 as an independent predictor of prostate cancer progression will be realised within a clinical setting.

3.3.3 Immunohistochemical Analysis Identifies Co-expression of Targets

Cyclin D1 is a critical regulator of G_1 phase progression in the cell cycle (Cordon-Cardo *et al.,* 1995). The cyclin D1 gene is located on a region of chromosome 11 containing the breakpoint of the t(11;14) translocation in B cell lymphomas, known as *bcl1.* Prior to cloning of the cyclin D1 gene, amplification of this region had been identified in multiple cancer types, including head and neck and breast (Ali *et al.,* 1989; Berenson *et al.,* 1989). Following the identification of cyclin D1 as the *bcl1* oncogene, its amplification and overexpression has been reported in additional cancer types, such as non-small cell lung cancer (NSCLC) (Betticher *et al.,* 1996). Overexpression of this protein has also been implicated in the evolution of hormone-refractory prostate cancer (Chen *et al.,* 1998).

Chen *et al.,* (1998) investigated the effects of elevated cyclin D1 expression on human prostate cancer progression through the transfection of androgen-sensitive LNCaP cells with a retroviral vector containing cyclin D1 cDNA. This study demonstrated that cyclin D1-transfected cells possessed a larger fraction of cells in the S-phase and a lower growth

factor requirement. These cells also grew more robustly in androgen-free medium. Furthermore, cyclin D1-transfected clones generated tumours more rapidly than control and parental cells. In addition, these tumours were found to be refractory to androgen ablation by castration. These findings indicate that cyclin D1 overexpression alters the proliferative properties of malignant cells, increasing their tumorigenicity and decreasing the requirement for androgen stimulation. Thus, there is much evidence to suggest that cyclin D1 overexpression may represent a significant oncogenic event in the progression of prostate cancer to androgen independence.

While our findings did not indicate a significant association between high cyclin D1 expression and tissue type (indolent vs. aggressive) based on our division criteria, a significant interrelationship was identified between cyclin D1 overexpression and both Ki-67 and ERG. These findings closely parallel those of previous studies, indicating that a strong association exists between the elevated cyclin D1 phenotype and a high Ki-67 proliferation index (Chen *et al.,* 1998; Drobnjak *et al.,* 2000). Drobnjak *et al.,* (2000) sought to further investigate the relationship between cyclin D1 overexpression and the development of androgen-refractory prostate cancer through the examination of cyclin D1 patterns in prostate carcinoma specimens derived from differential time points representing the natural biological continuum of the disease. This study reported that a larger proportion of androgen-independent metastatic prostate cancer cases exhibited an increased proliferative activity compared to primary tumours. Interestingly, this enhanced proliferative index was significantly associated with elevated levels of cyclin D1.

In some cases, enrichment of cyclin D1 has been associated with an increased rate of disease progression. For example, in hepatocellular carcinoma, overexpression of cyclin D1 can be dramatic and predisposes to a more aggressive disease phenotype (Nishida *et al.,* 1994). Overexpression of cyclin D1 has also been demonstrated in the majority of invasive lobular mammary carcinomas and the marked absence of cyclin D1 expression in non-invasive cells indicates that elevated levels of this protein are involved in progression to the invasive form of this malignancy (Oyama *et al.,* 1998). In contrast to these findings, overexpression of cyclin D1 in primary prostate tumours is postulated to be a rare event. Gumbiner *et al.,* (1999) analysed the expression of this oncogene in 96 human prostate tumours, 15 cases of benign prostatic hyperplasia, 4 prostate cancer cell lines and 3 xenografts. Their findings demonstrated that only 4.2% of prostate tumours overexpressed cyclin D1, while levels of this transcript were found to be normal in cell lines, BPH cases and xenografts. This data differs significantly from the findings of our study, which demonstrated that 25 of 47 prostate tumours analysed (53.19%) expressed elevated levels of cyclin D1. The reasons behind this heterogeneity in cyclin D1 transcript levels remain unclear but it is

interesting to note, that cyclin D1 overexpression is not simply a uniform hallmark of the tumorigenic state indicating that this event may hold significant clinical consequences in terms of disease progression in a subset of prostate cancers. Further evidence indicating that abnormal cyclin D1 expression is a driver of prostate cancer progression, has come from a recent study by Ju *et al.,* (2014), which demonstrated that a cyclin D1-induced gene signature could predict biochemical recurrence in human prostate tumour specimens.

Taken together, these findings suggest that cyclin D1 overexpression is a critical oncogenic event in prostate tumorigenesis, which profoundly alters proliferation control as demonstrated by a reciprocal elevation in Ki-67 proliferation index and may infact be related to the subsequent development of bone metastases. Our findings support those of previous studies, which have identified a definitive positive relationship between high cyclin D1 expression and increased proliferation in a subset of prostate carcinomas. Given the abundance of evidence implicating this phenotype as a critical event in the evolution of multiple cancer types, a retrospective reconciliation of this data with the biochemical status of patients within this cohort will be warranted in the future, in order to determine whether elevated cyclin D1 expression is infact a reliable predictor of poor clinical outcome.

As previously mentioned, the current study also identified a significant association between high cyclin D1 expression and positive ERG status. Our results suggested that the odds of being ERG positive are four times greater in those who also overexpress cyclin D1. Furthermore, this phenomenon was found to be age dependent. Our data demonstrated that older age is less associated with ERG positivity. Indeed the current analysis suggests that for every year increase in age, the odds of possessing elevated ERG levels decrease by 0.871. This age association is not entirely novel as TMPRSS2-ERG fusions have recently been shown to be strongly linked to young patient age, particularly in low-grade prostate cancer (Steurer *et al.,* 2014). In an attempt to understand how age-dependent molecular features influence cancer phenotype Steurer *et al.,* (2014) performed a next-generation sequencing analysis of early-onset prostate cancer, the results of which demonstrated that the incidence of TMPRSS2-ERG fusions decreases with increasing patient age. Interestingly, this phenomenon was found to be limited to low-grade cancers of Gleason ≤3+4. It may be hypothesised that this anomaly occurs as a direct consequence of high testosterone levels in younger men as cell line studies have demonstrated that elevated androgen levels are sufficient to induce spontaneous TMPRSS2-ERG fusions (Mani *et al.,* 2009). Furthermore, the observed absence of an age association with ERG status in high-grade prostate cancers suggests that ERG-positive cancers may progress to high-grade tumours by androgen-independent mechanisms. Thus, it may be postulated that androgen-

driven translocation events in young patients specifically predispose low-grade prostate carcinoma.

Studies have demonstrated that the introduction of the ERG gene fusion into primary or immortalised benign prostate epithelial cells has been demonstrated as insufficient to increase cellular proliferation (Tomlins *et al.,* 2008). It is noteworthy, that our data indicated a significant association between an elevated proliferative phenotype and ERG positivity as it may be possible that an increased rate of proliferation coupled with a background of genetic instability could facilitate a more aggressive disease phenotype in certain cases. Again, as this archival cohort matures, post-operative follow-up data will be crucial in determining the significance, if any, of these findings.

3.4 Conclusion

In recent years, the strengthening of discovery technologies has facilitated the identification and publication of thousands of putative biomarkers. However, the translation of these biomarkers from discovery to clinical practise is a process complicated by a multitude of scientific limitations (Drucker *et al.,* 2013). The current study was undertaken to explore the miRNA expression profile of an archival prostatectomy cohort drawn from the prostate cancer research consortium's archival bioresource. It was hypothesised that a differential miRNA expression profile would distinguish putatively indolent prostate carcinoma from intrinsically aggressive cases. However, the results of this analysis were equivocal and inconsistent, which highlighted many of the practical issues which routinely confound biomarker research studies. The heterogeneous phenotype and protracted natural progression of prostate cancer ensures that successful biomarker studies will necessitate a large cohort of well-stratified patients. In addition, the optimal selection of patients also requires careful consideration and is often problematic due to the lack of sufficient clinical specimens to meet study criteria. As previously discussed, many promising biomarkers identified through immunohistochemical analysis have failed to replace existing clinical tests, which is a consequence of disparate methodologies and the application of conflicting cutoffs to measure positivity. However, the incorporation of these auspicious markers into a refined multiparametric predictive panel may yield the appropriate sensitivity required for clinical use. In this study, we have demonstrated that cyclin D1 shows an interesting expressional dynamic with both Ki-67 and ERG. It has been postulated that abnormal elevation of this crucial cell cycle control gene induces progression of prostate cancer to androgen-refractory disease. The preliminary data presented here in combination with data from the literature leads this postulation to warrant further examination (Drobnjak *et al.,* 2010; Chen *et al.,* 1998). In summary, this small study has demonstrated that a number of practical limitations

must be overcome in order to successfully elucidate the fundamental physiological differences between insignificant and aggressive prostate cancer.

Expression Profiling of a Prostate Cancer Research Consortium (PCRC) Patient Cohort

Chapter 4

Chapter 4. Expression Profiling in a PCRC Patient Cohort

4.1 Introduction

4.1.1 The Prostate Cancer Research Consortium

The Prostate Cancer Research Consortium (PCRC) was established late in 2003 with the overarching aim of creating a collaborative multi-institutional infrastructure in order to coordinate translational prostate cancer research in Ireland. The research focus of the PCRC has been driven by the many challenges, which currently encumber the effective clinical management of prostate cancer. A startling statistic released by the National Cancer Registry of Ireland predicted a 275% increase in prostate cancer incidence between 2006 and 2016 (NCRI, 2006). While this projected increase likely represents an artefact of opportunistic PSA screening, particularly in men with no disease symptoms, the inability to effectively manage these patients remains a clinical dilemma. This rise in cases underscores the emphatic requirement for refined biomarkers to improve prostate cancer risk stratification, thereby abrogating the overtreatment of clinically insignificant prostate cancer and effectively identifying those patients who are at the greatest risk of disease progression. This is a technically challenging objective, which remains the cornerstone of the prostate cancer research consortium's founding.

The PCRC comprises three clinical sites; the Mater Misericordiae University Hospital, St. James's Hospital and Beaumont Hospital and four research institutions; the Conway Institute (University College Dublin), Institute of Molecular Medicine (Trinity College Dublin), RCSI-Education and Research Centre (Royal College of Surgeons in Ireland) and National Centre for Sensor Research (Dublin City University). The first major aim of the PCRC was the establishment of a comprehensive biobank of clinically relevant patient samples. To date, the PCRC-dedicated bioresource has collected radical prostatectomy tissue, plasma, serum and urine from over 600 patients, and has concomitantly created a rich database of post-operative follow-up data in the intervening years. This bioresource of patient material currently fuels the major research objective of the PCRC; to apply genomic, transcriptomic and proteomic technologies to the identification of novel prognostic biomarkers to aid risk stratification and better inform clinical decisions.

In 2011, a major collaborative project was undertaken to harness the prostate cancer research consortium's extensive resources, in order to investigate predictive biomarkers in genomics (mRNA and miRNA expression signatures, hypermethylation), proteomics (glycosylation, PSA isoforms) and pathological image analysis across multiple biological

matrices. The fundamental objective of this project was to employ a novel data integration approach to yield a refined panel of predictive biomarkers for interrogation in pre-operative urine and biopsy material, which would ultimately challenge the current inability to delineate those patients with intrinsically aggressive disease. While a number of critical biomarker studies have identified signatures, which molecularly characterise prostate cancer, as yet no effective prognostic signature has entered clinical utility. The novel data integration approach adopted by the PCRC collaborative project differs from the biomarker studies which have come before it in that it is the first of its kind to perform exhaustive genomic, transcriptomic and proteomic profiling of the same well-defined sample set. It is hoped that this comprehensive approach will be more successful than conventional single feature algorithms in identifying a signature of aggressive disease. To this end, a leading aim of this work was to perform a large portion of the genomic aspect of the PCRC integrated effort.

4.1.2 Integrated miRNA/mRNA Signature

The importance of miRNA/gene expression profiling in the setting of biomarker discovery has been discussed at length throughout this thesis (Chapter 1; 1.6.2, 1.7.3 and Chapter 3). Increasingly, miRNAs are recognised as clinically relevant, robust biomarkers which can be successfully quantified in blood and other bodily fluids, thus supporting their application as non-invasive tests (Mitchell *et al.,* 2008). In addition, the availability of high-throughput microarray technology allows the profiling of thousands of genes, which has led to innumerable molecular profiling studies of prostate cancer (Dhanasekaran *et al.,* 2001; Luo *et al.,* 2001, Welsh *et al.,* 2001; Lapointe *et al.,* 2004). While these studies have increased our understanding of tumorigenesis through the identification of differentially expressed genes between normal and tumour prostate tissue, they have not yet identified a robust biomarker to better the current clinical parameters utilised to predict disease progression. Thus patient-tailored therapy remains an unachieved goal. The failure of these studies to yield clinically feasible results is likely owing to a number of unavoidable study limitations including sample size and the implementation of inappropriate surrogate endpoints (Sboner *et al.,* 2010). The coordinated multidisciplinary research model of the PCRC was devised in an attempt to overcome these challenges.

As previously mentioned, many gene expression profiling studies have yielded highly complex molecular signatures and computational strategies have been employed to reduce these gene lists to smaller non-redundant predictive gene panels. For example, a study by Ramaswamy *et al.,* (2003) has identified a 17-gene signature of cancer metastasis capable of distinguishing primary from metastatic adenocarcinomas. Thus, there is compelling evidence to indicate that clinically relevant predictive molecular signatures of cancer

progression are composed of refined gene panels, once redundant targets have been removed from the initial high-throughput analyses (Bismar *et al.*, 2006). In recent years, meta-analyses of microarray studies have been employed to make sense of the increasing number of molecular profiling datasets available. This general bioinformatic approach, which integrates various sources of genome-wide expression data, has already proven successful in the classification of diffuse large B-cell lymphomas (Lossos *et al.*, 2004).

A meta-analysis previously performed within the laboratory has identified a number of gene expression studies, which are of particular relevance to prostate cancer progression (Figure 4.1). Specifically a 17 gene signature identified by Nakagawa et al., (2008) which predicts systemic progression following primary treatment for prostate cancer, a 7 gene signature identified by Laxman et al., (2008) for the early detection of prostate cancer in urine and finally a 9-gene prognostic signature specifically for use in formalin-fixed paraffin-embedded samples (Sorensen et al., 2010). A major objective of this project was to build upon the results of previous efforts to identify predictive biomarkers by incorporating these gene sets into an inductive computational search for putative prognostic biomolecular markers.

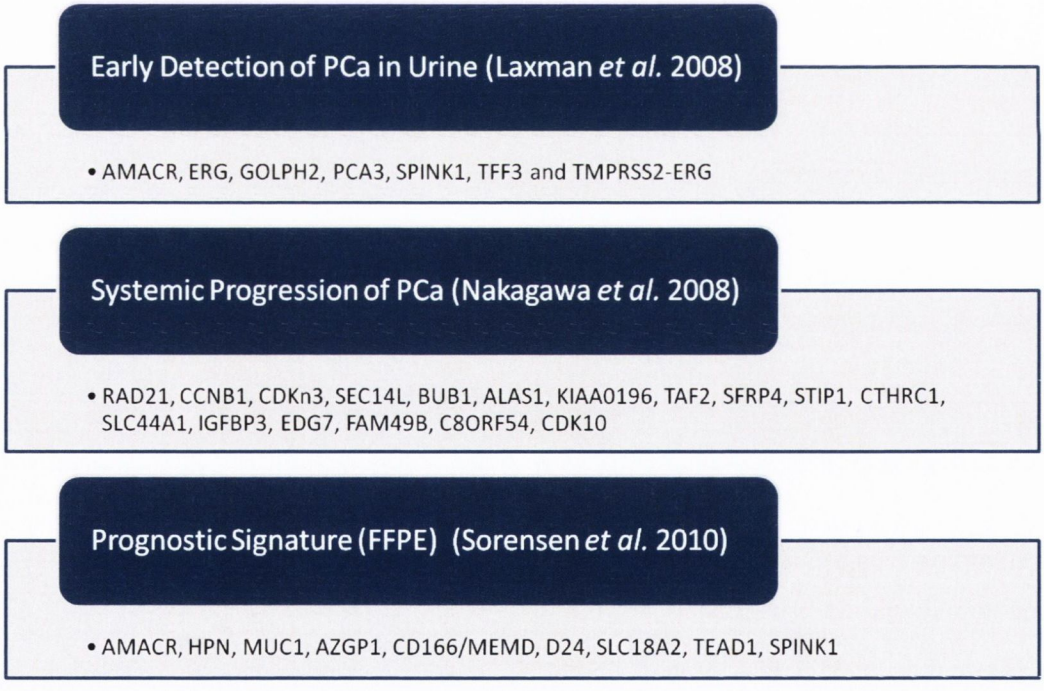

Figure 4.1 *Clinically relevant gene signatures identified from the literature.*

These gene sets were identified through an extensive literature search with the intention of performing a comprehensive bioinformatic data analysis to yield a focussed, clinically relevant gene panel for interrogation in the PCRC cohort.

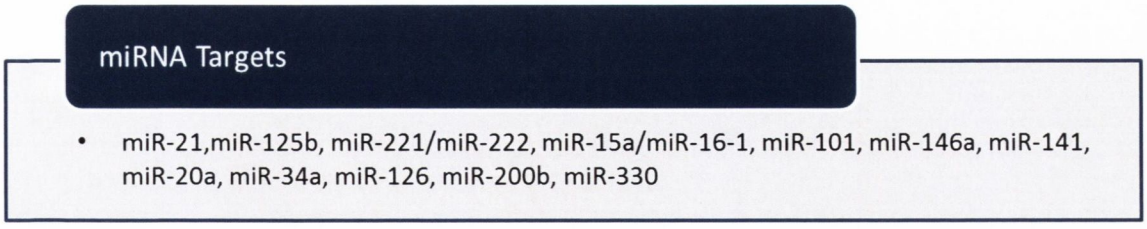

Figure 4.2 *Biologically relevant miRNA panel for interrogation in the PCRC cohort.*

This miRNA panel was identified from a composite expression profiling/literature search previously performed within the laboratory (Chapter 3).

4.1.3 Hypothesis and Project Aims

Currently, prostate cancer prognostication and patient stratification are reliant upon the clinical and histopathological parameters of PSA measurement and Gleason scoring (Hughes *et al.*, 2005). Unfortunately, these prognostic indicators are not sufficiently accurate to predict disease progression and appropriately inform clinical decision making. This paucity of accurate testing coupled with the discernible heterogeneous nature of prostate cancer has led to the overtreatment of insignificant disease. Much evidence has demonstrated that comprehensive studies of gene and miRNA expression provide an insight into the pathologic behaviour of human cancer by identifying differences in expression patterns between disease subtypes. It has been postulated that such differences may be harnessed to construct a molecular signature of disease characteristics (Lapointe *et al.*, 2004). With this in mind, the hypothesis of this work was that there exists a unique integrated mRNA/miRNA expression signature which may, at least in part, molecularly delineate clinically indolent from aggressive prostate neoplasms and that the multi-disciplinary integrated research approach adopted by the PCRC would be more successful in identifying a clinically relevant biomarker panel than previous studies. Furthermore, it has been postulated that self renewing cancer stem cells are heavily implicated in the progression of prostate cancer and the development of treatment resistance. The discovery that intrinsic characteristics of embryonic stem cells such as self renewal are subject to intense miRNA regulation has led to the hypothesis that the dysregulation of cancer stem cell-associated miRNAs contributes to tumour aggressiveness through the uninhibited expansion of the cancer stem cell population.

Thus, the primary objectives of these investigations were to;

- Identify a panel of gene targets for interrogation in a defined clinical cohort of patients with indolent, significant and aggressive prostate cancer through a preliminary bioinformatic pathways analysis utilising the results of a pilot global miRNA expression analysis performed by our collaborators in the Harvard School of Public Health (HSPH, Boston, MA).

- Examine the expression of our miRNA/gene panel in treatment-naive radical prostatectomy tissue samples from the defined PCRC cohort using optimised TaqMan microfluidic cards.

- Investigate the expression of four critical miRNAs which have previously been implicated in the modulation of embryonic stem cell characteristics and which have been identified as deregulated in prostate cancer tissue; let-7a, let-7b, miR-143 and miR-145 (Kong *et al.,* 2012, Huang *et al.,* 2012).

4.2 Results

4.2.1 Prostate Cancer Research Consortium Patient Cohort

The patient cohort for use in this study was drawn from the prostate cancer research consortium bioresource of treatment-naive radical prostatectomy (RP) specimens excised in multiple clinical institutions between 2006 and 2012 (Table 4.1). Patient samples were collected according to standard operating procedures (SOPs) through a single research nurse. Ethical consent was granted from the Mater Misericordiae University Hospital, Beaumont Hospital and St. James's Hospital ethics committee. Samples were de-identified by assignment of a PCRC number.

Epstein's criteria were used to define patients as having indolent, significant or aggressive prostate cancer (Epstein *et al.,* 1994). Indolent prostate cancer was defined as tumour volume $<0.5cm^3$, organ-confined disease and no Gleason patterns 4 or 5 present upon pathological examination of RP specimens. Significant disease was defined as tumour volume $>0.5cm^3$, organ-confined disease and the presence of Gleason pattern 4 (maximum). Aggressive prostate cancer was defined as Gleason patterns 4 or 5 and the presence of extracapsular extension/non organ-confined disease. 46 of the total 682 patients recruited into the PCRC bioresource to date were classified as insignificant. Subsequently, 56 PSA and age-matched significant and aggressive patients were chosen for inclusion in the cohort (Table 4.1).

Archival formalin-fixed paraffin-embedded blocks were collected from multiple sites and sections were histologically prepared for review by a pathologist. The appropriate tumour lesions were marked on haematoxylin and eosin (H&E)-stained slides and the corresponding tissue was macrodissected from a whole section on a slide (4 slides per patient) for RNA extraction. Upon pathological review, a number of cases were found to possess no tumour/insufficient biomaterial, and so were removed from the final cohort analysed in this study (Table 4.2).

	Full Data (n = 158)	Indolent (n = 46)	Significant (n = 56)	Aggressive (n = 56)	*p*
Age (y)					
Mean (Median)	60.6(61)	59.8(60)	59.8(60)	61.9(62.5)	*0.087*
Range	45-74	49-70	48-73	45-74	
Pre-PSA (ng/ml)					
Mean (Median)	6.89(6.45)	6.35(6)	6.72(6.34)	7.49(7)	*0.168*
Range	0.7-18.7	0.7-12.3	2.4-14.7	2.3-18.7	
Clinical Stage, DRE (%)					
T1c	79(50)	26(57)	31(55)	22(40)	*0.082*
T2a	36(23)	5(11)	14(25)	17(30)	
Not Reported	43(27)	15(32)	11(20)	17(30)	
Biopsy GS (%)					
≤6	70(44)	40(93)	22(40)	9(18)	*0.000*
3 + 4 = 7	60(34)	3(7)	25(45)	22(42)	
4 + 3 = 7	17(11)	0(0)	7(13)	10(20)	
≥8	11(7)	0(0)	1(2)	10(20)	
Prostatectomy Stage (%)					
OC	102(65)	46(100)	56(100)	0(0)	*0.000*
NOC	56(35)	0 (0)	0 (0)	56(100)	
Prostatectomy GS (%)					
≤6	47(30)	46(100)	1(2)	0 (0)	*0.000*
3 + 4 = 7	72(46)	0 (0)	46(82)	26(46)	
4 + 3 = 7	26(16)	0 (0)	5(9)	21(38)	
≥8	13(8)	0 (0)	4(7)	9(16)	

Table 4.1 *Clinicopathological characteristics of the prostate cancer research consortium patient cohort.*

The initial patient cohort was identified from the PCRC bioresource of 682 patient specimens based on a defined set of clinical disease parameters and divided into three disease subtypes; Insignificant/Indolent, Significant and Aggressive. Upon histopathological review of the slides a number of cases were found to contain no tumour area/insufficient material and as a result were removed from the final cohort. GS: **G**leason **S**core, OC: **O**rgan **C**onfined, NOC: **N**on-**O**rgan **C**onfined.

	Full Data (n = 120)	Indolent (n = 23)	Significant (n = 50)	Aggressive (n = 47)	*p*
Age (y)					
Mean (Median)	60.7(60.5)	59.2(59)	59.8(60)	62.4(63)	*0.091*
Range	48-74	49-68	48-73	49-74	
Pre-PSA (ng/ml)					
Mean (Median)	7.8(6.5)	6.4(5.6)	6.5(6.09)	7.75(7.25)	*0.174*
Range	1.2-18.7	1.2-12	2.4-14.7	3.1-18.7	
Clinical Stage, DRE (%)					
T1c	69(60)	19(82)	31(62)	20(42)	*0.087*
T2a	25(39)	4(17)	10(20)	15(31)	
Not reported	26(21)		9(18)	12(24)	
Biopsy GS (%)					
≤6	49(40)	20(86)	18(36)	8(17)	*0.000*
3 + 4 = 7	43(35)	3(13)	25(50)	22(46)	
4 + 3 = 7	17(14)	0(0)	6(12)	7(14)	
≥8	11(9)	0(0)	1(2)	10(21)	
Prostatectomy Stage (%)					
OC	73(60)	23(100)	50(100)	0 (0)	*0.000*
NOC	47(39)	0 (0)	0 (0)	47(100)	
Prostatectomy GS (%)					
≤6	23(19)	22(100)	1(2)	0 (0)	*0.000*
3 + 4 = 7	63(52)	0 (0)	41(82)	21(44)	
4 + 3 = 7	24(20)	0 (0)	5(10)	19(40)	
≥8	10(8)	0 (0)	3(6)	7(14)	

Table 4.2 *Clinicopathological characteristics of the final cohort used in this study.*

The final cohort used in this study was refined from 158 total cases to 120 due to insufficient tumour material and no tumour present upon pathological examination. Indolent samples comprised the smallest patient cohort as tumour foci for macrodissection were too small in a large proportion of cases. GS: **G**leason **S**core, OC: **O**rgan **C**onfined, NOC: **N**on-**O**rgan **C**onfined.

4.2.2 Selection of a Gene Target Panel

The preliminary 33-gene panel for interrogation in this study was compiled by a comprehensive review of the literature previously performed within our laboratory (Figure 4.1). In order to refine this gene list and create a more focussed panel for investigation, a bioinformatic computational target validation was performed, incorporating data from a pilot global miRNA expression analysis performed by our collaborators in the Harvard School of Public Health (HSPH).

This study utilised the high-throughput OpenArray® system. OpenArray® technology is a real-time PCR based system, which minimises reagent use and allows the high-throughput analysis of gene and miRNA expression. Each OpenArray® chip can accommodate the same quantity of samples as 8 x 384-well cards. Global miRNA expression was examined in a small sample set of 7 formalin-fixed paraffin-embedded patient specimens (both tumour and normal for each patient) of varying Gleason Scores and TMPRSS2-ERG-fusion status (Table 4.3). Tissue was extracted via laser capture microdissection (LCM) and a preamplification step was incorporated into the protocol to circumvent any issues with low sample input. miRNA expression analysis was compared between Gleason score 6 vs. 8 cases.

Following endogenous control normalisation, five miRNAs were identified as significantly differentially expressed between Gleason 6 and Gleason 8 cases; miR-205 (p=0.008), miR-31 (p=0.01), miR224 (p=0.01), miR-222 (p=0.01) and miR-31* (p=0.02) (Courtesy of Irene Shui, HSPH Boston, USA). As these results were preliminary and based upon a small sample size, the top fifty differentially expressed miRNAs were chosen from this study and used as a supplement to our own miRNA panel (Chapter 3) (Table 4.4).

Each selected miRNA was used to query two separate databases of experimentally validated miRNA: target interactions (MTIs) (miRWALK and miRTarBase). The results of this MTI analysis were cross-referenced with our 33-gene panel. Eleven genes within the panel were found to have a known MTI with one or more of the 65 miRNAs analysed. These 11 genes were taken forward for analysis in the PCRC cohort (Table 4.5).

Case	Tissue	Gleason Score	TMPRSS2/ERG Status
1	Tumour	8	Positive
	Normal	N/a	
2	Tumour	8	Not Reported
	Normal	N/a	
3	Tumour	6	Positive
	Normal	N/a	
4	Tumour	6	Negative
	Normal	N/a	
5	Tumour	8	Positive
	Normal	N/a	
6	Tumour	7	Negative
	Normal	N/a	
7	Tumour	6	Negative
	Normal	N/a	

Table 4.3 *Pilot global miRNA expression analysis sample set.*

Harvard collaborators performed a pilot global miRNA expression analysis of n=7 patient specimens, with both tumour and normal tissue for each patient.

Differentially Expressed miRNAs				
miR-205	miR-1274b	miR-143	miR-23b	miR-27b
miR-31*	miR-204	miR-145*	miR-328	miR-135a
miR-224	miR-181c	miR-145	miR-152	miR-503
miR-222	miR-34c	miR-339-5p	miR-184	miR-330
miR-31	miR-146b-3p	miR-296	miR-214	miR-133a
miR-410	miR-376c	miR-222*	miR-624	miR-193a-5p
miR-455-3p	miR-383	miR-21*	miR-543	miR-886-5p
miR-455	miR-422a	miR-133b	miR-130a	miR-193b
miR-26a	miR-642	miR-101	miR-125b-2*	miR-635
miR-221	miR-29a*	miR-409-3p	miR-378	miR-411

Table 4.4 *Table of fifty differentially expressed miRNAs identified in the pilot global miRNA expression analysis.*

This list was used as a supplement to our own miRNA panel. A small degree of redundancy was noted between the two miRNA panels. *indicates miRNA isoform

	CCNB1	AMACR	SPINK1	SFRP4	TMPRSS2	MUC1	ERG	STIP1	IGFBP3	FAM49B	ALCAM
miR-125b						X			X		
miR-141		X									
miR-15a	X		X								
miR-16-1	X		X					X			
miR-200b	X										
miR-20a	X										
miR-21						X					
miR-221		X			X		X				
miR-26b				X						X	
miR-31				X							
miR-34a	X										
miR-193b											X

Table 4.5 *Correlation matrix of the final 11-gene panel and the corresponding validated miRNA:target interactions.*

Bioinformatic target validation analysis yielded a panel of 11 genes, whereby each gene possessed one or more miRNA-target interaction with a candidate from the supplemented panel.

4.2.3 Gene Expression Profiling in a Defined Clinical Cohort

384-well microfluidic qRT-PCR cards were used to examine the expression of our final gene panel in the 120-patient prostate cancer research consortium cohort. To correct for systematic variables, samples were normalised to the expression of the endogenous control gene GAPDH. A C_T cutoff of 37 was implemented whereby cycle threshold values of 37-40 were considered as absent expression.

A three-way ANOVA was performed to determine statistically significant differences in expression of each gene across the three tumour classifications. Three genes were identified as statistically significantly differentially expressed across the three subgroups, IGFBP3 (p= 0.04), MUC1 (p= 0.02) and SFRP4 (p=0.01) (Figure 4.3, Figure 4.4).

Pairwise analysis was then performed between individual groups; Indolent vs. Aggressive, Aggressive vs. Significant and Indolent vs. Significant. Significant differential gene expression patterns were identified between the aggressive and significant patient groups. AMACR (p= 0.03), CCNB1 (p= 0.01) and FAM49B (p= 0.02) were found to be statistically significantly upregulated in the aggressive group when compared to significant (Figure 4.5, Figure 4.6). In addition, IGFBP3 (p = 0.02), MUC1 (p= 0.02) and SFRP4 (p= 0.01) were also found to be statistically significantly upregulated in the aggressive group compared to significant. No other genes within the panel investigated were found to exhibit significant differential expression patterns.

Kernel density estimation (KDE) is a non-parametric method to estimate the probability density function of a random variable. KDE was employed to visually assess the distribution patterns of the RQ values for each tumour category (Figure 4.9).

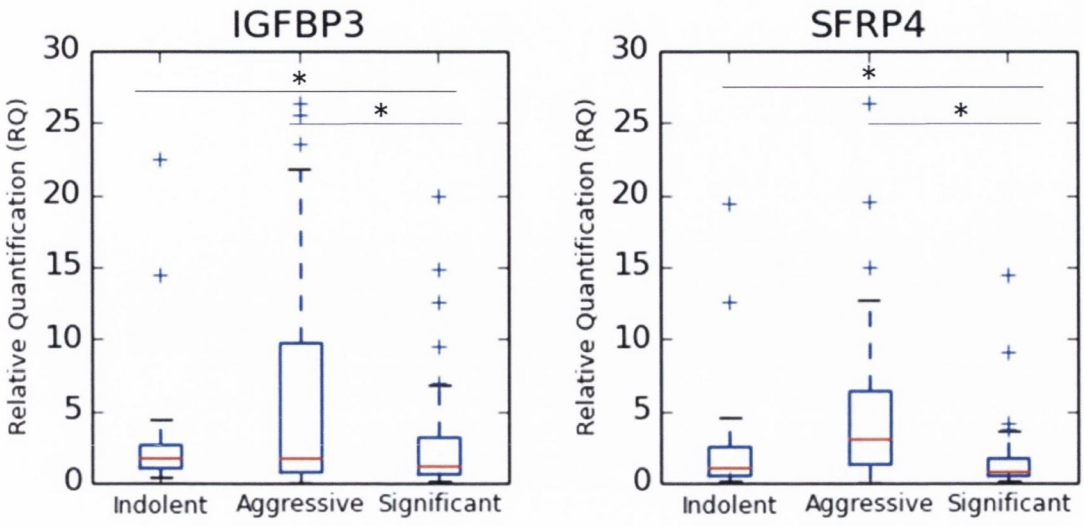

Figure 4.3 *Boxplots of relative quantification (RQ) of change in expression of IGFBP3 and SFRP4 across tissue cohorts.*

IGFBP3 and SFRP4 were found to be statistically significantly differentially expressed across the three tumour classifications. Pairwise analysis also indicated that the expression of IGFBP3 and SFRP4 was statistically significantly upregulated in the aggressive group when compared to the significant. * indicates statistical significance ($p < 0.05$) between groups as identified by ANOVA and Mann-Whitney U Test. + indicates outliers.

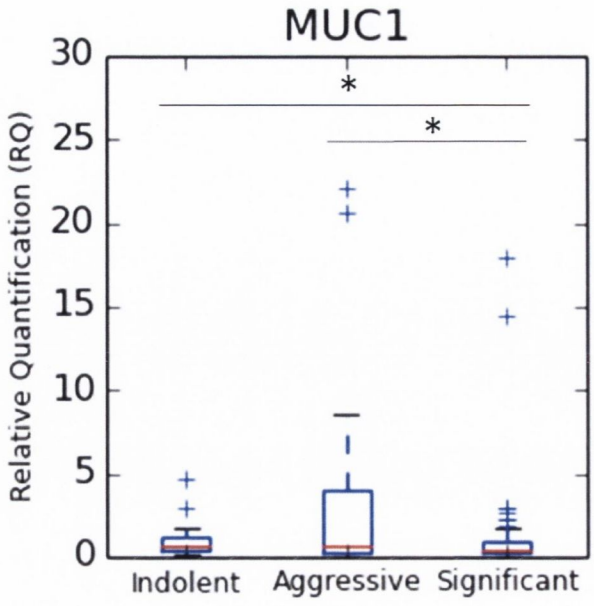

Figure 4.4 *Boxplot of relative quantification (RQ) of change in expression of MUC1 across tissue cohorts.*

MUC1 was found to be statistically significantly differentially expressed across the three tumour classifications. Pairwise analysis also indicated that expression of MUC1 was statistically significantly upregulated in the aggressive group when compared to the significant. * indicates statistical significance ($p < 0.05$) between groups as identified by ANOVA and Mann-Whitney U Test. + indicates outliers.

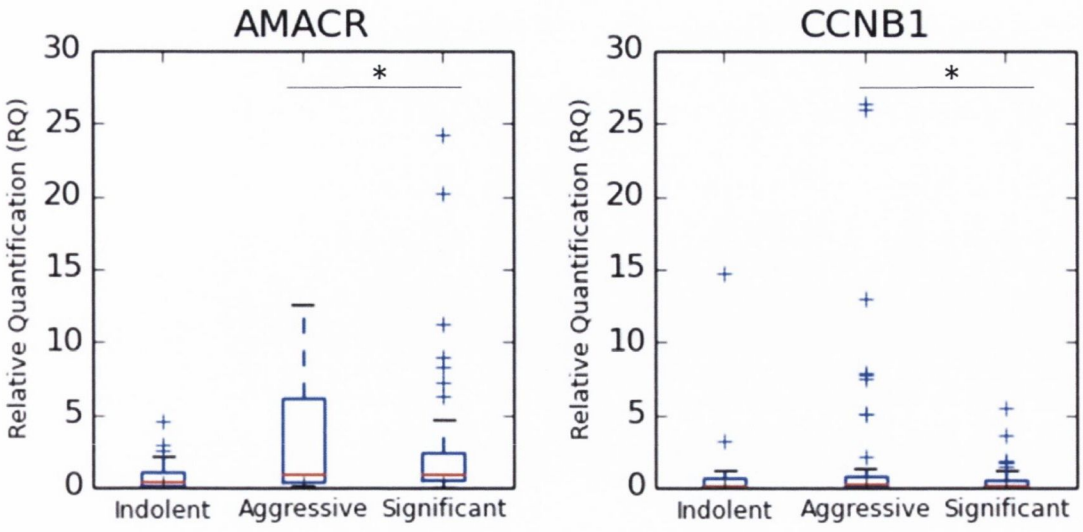

Figure 4.5 *Boxplots of relative quantification (RQ) of change in expression of AMACR and CCNB1 across the tissue cohorts.*

The expression of AMACR and CCNB1 was found to be statistically significantly upregulated in the aggressive cohort when compared to the significant group. * indicates statistical significance ($p < 0.05$) between groups as identified by Mann-Whitney U Test. + indicates outliers.

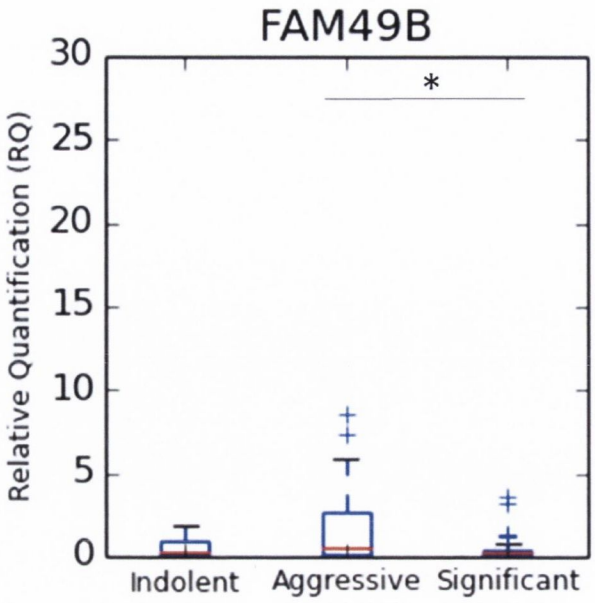

Figure 4.6 *Boxplot of relative quantification (RQ) of change in expression of FAM49B across the tissue cohorts.*

The expression of FAM49B was found to be statistically significantly upregulated in the aggressive cohort when compared to the significant group. * indicates statistical significance ($p < 0.05$) between groups as identified by Mann-Whitney U Test. + indicates outliers.

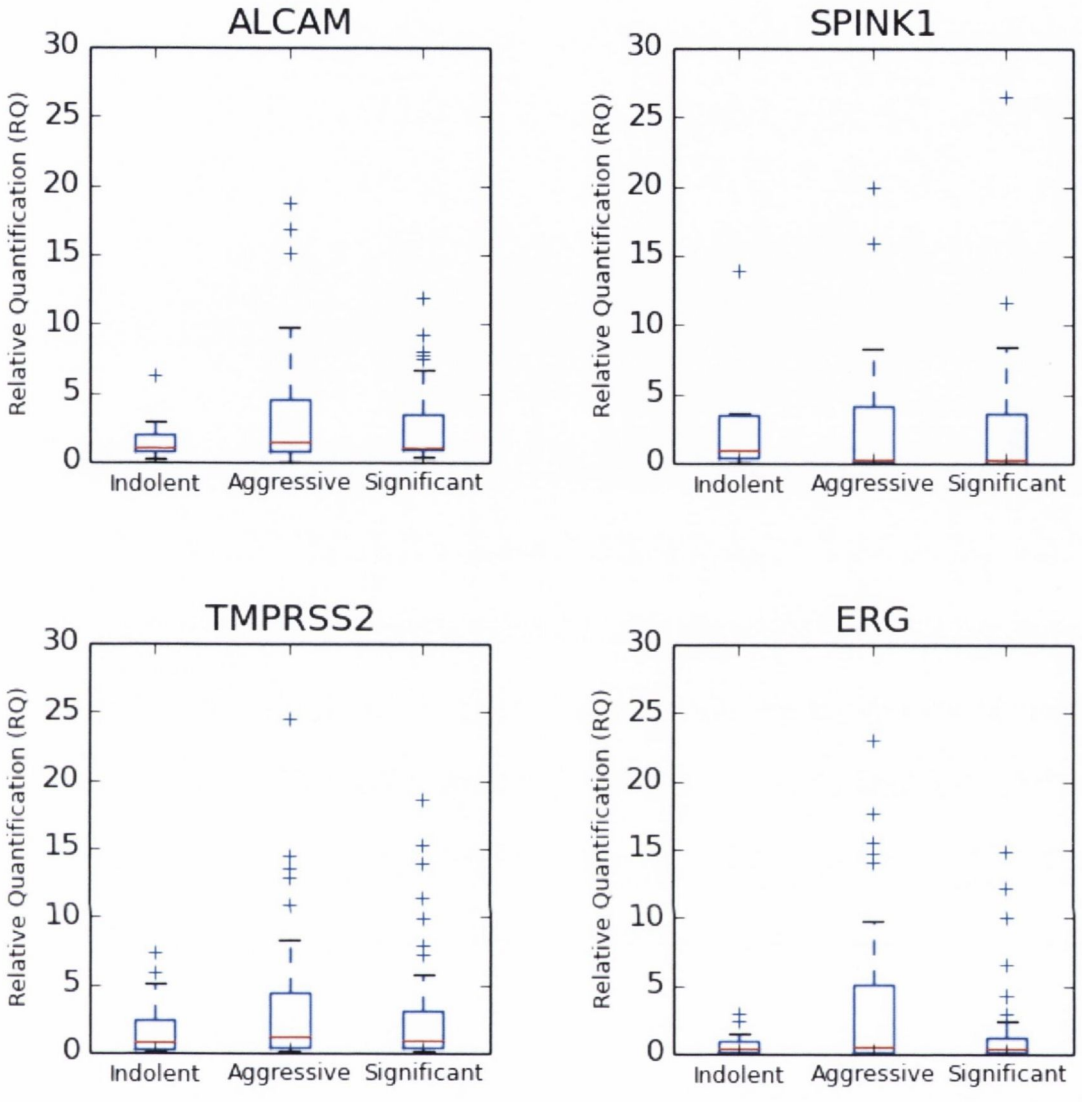

Figure 4.7 *Boxplots of relative quantification (RQ) of expression of ALCAM, SPINK1, TMPRSS2 and ERG across the tissue cohorts.*

No statistically significant difference in expression was identified. + indicates outliers.

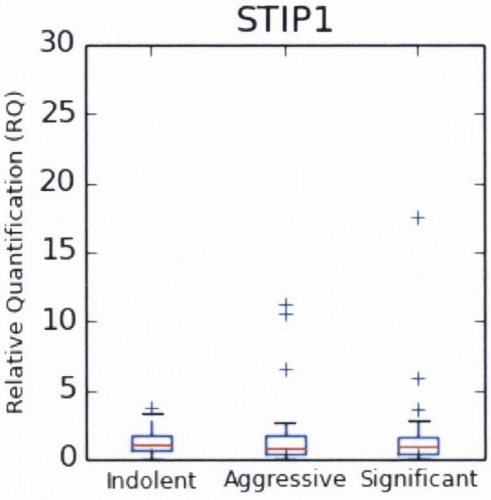

Figure 4.8 *Boxplot of relative quantification (RQ) of expression of STIP1 across the tissue cohorts.*

No statistically significant difference in expression was identified. + indicates outliers.

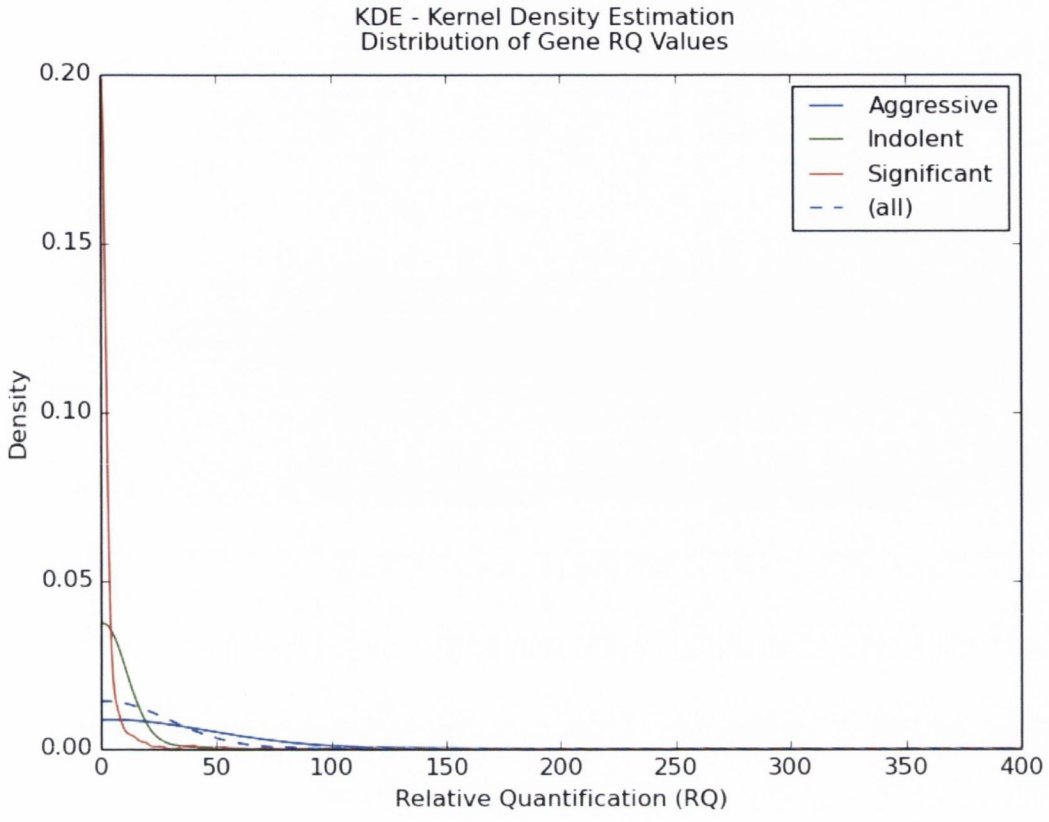

Figure 4.9 *Kernel density distribution plot of relative quantification (RQ) of gene expression.*

Kernel density estimation (KDE) was used to plot the probability density function of the relative quantification values of gene expression. The dashed line represents the data from the three cohorts represented as a whole. The distribution of the significant group (red) indicates that the probability of a large-fold change in expression is highest within this group.

4.2.4 miRNA Expression Profiling in a Defined Clinical Cohort

miRNA expression analysis was also performed using 384-well qRT-PCR microfluidic cards. Samples were normalised to the expression of the endogenous control; small nucleolar RNA U6 (snoRNA U6). A C_T cutoff of 37 was implemented whereby cycle threshold values of 37-40 were considered as absent expression.

A three-way ANOVA was performed to determine statistically significant differences in expression of each miRNA across the three tumour classifications. No miRNAs were identified as statistically significantly differentially expressed. Subsequent pairwise testing between individual groups identified no statistically significant differences in miRNA expression (Figure 4.10, Figure 4.11, Figure 4.12, Figure 4.13).

The relative quantification (RQ) values of miRNA expression were plotted using KDE and interesting differences in the distribution of the data were identified between the three tumour groups (Figure 4.14). KDE showed the probability of a uniformly large fold change in miRNA expression was highest in the aggressive group, while the indolent group was found to follow a similar pattern. The significant group was found to contain a larger degree of heterogeneity in RQ values.

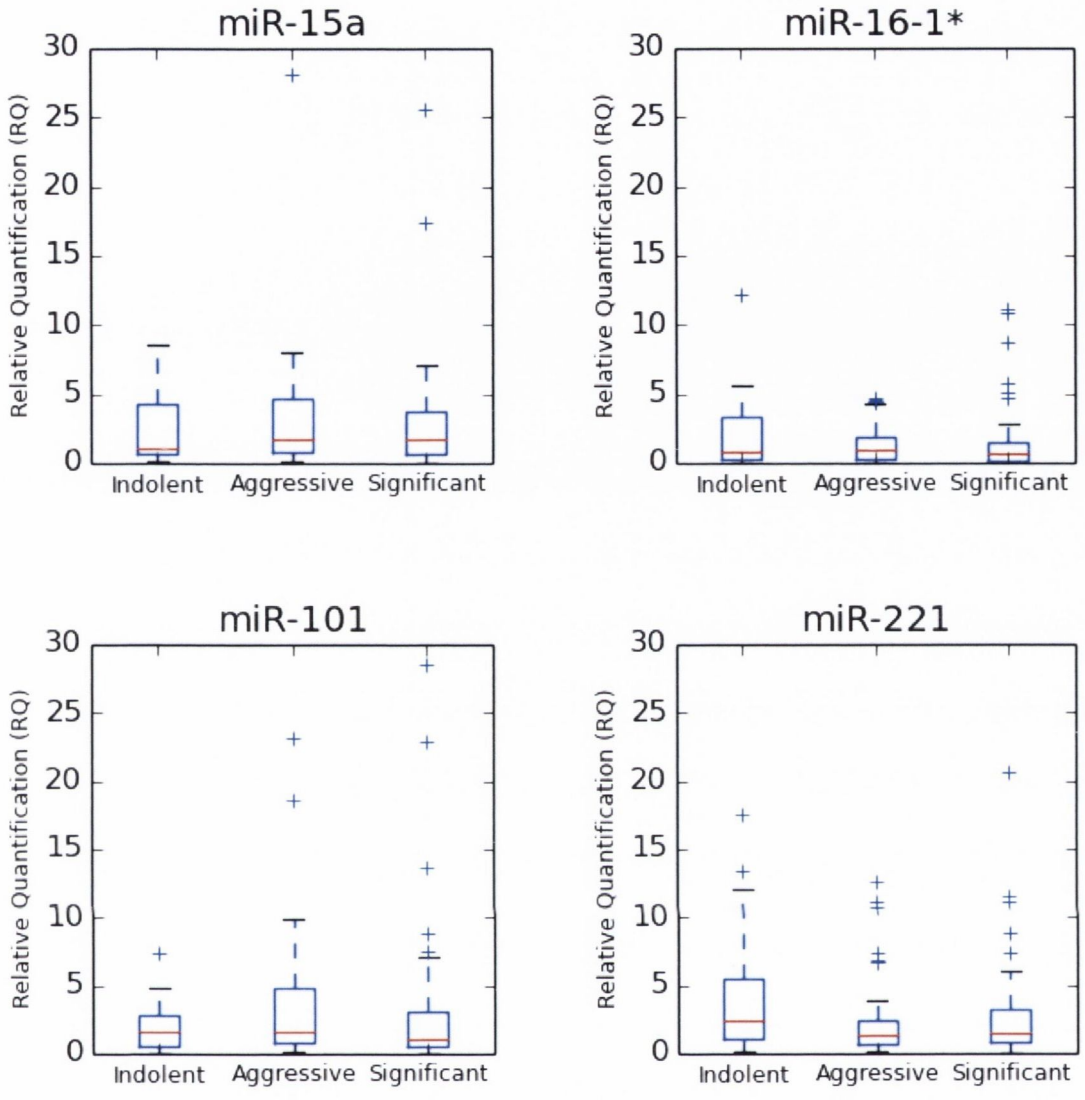

Figure 4.10 *Boxplots of relative quantification (RQ) of expression of miR-15a, miR-16-1*, miR-101 and miR-221 across the tissue cohorts.*

No statistically significant difference in expression was identified. + indicates outliers.

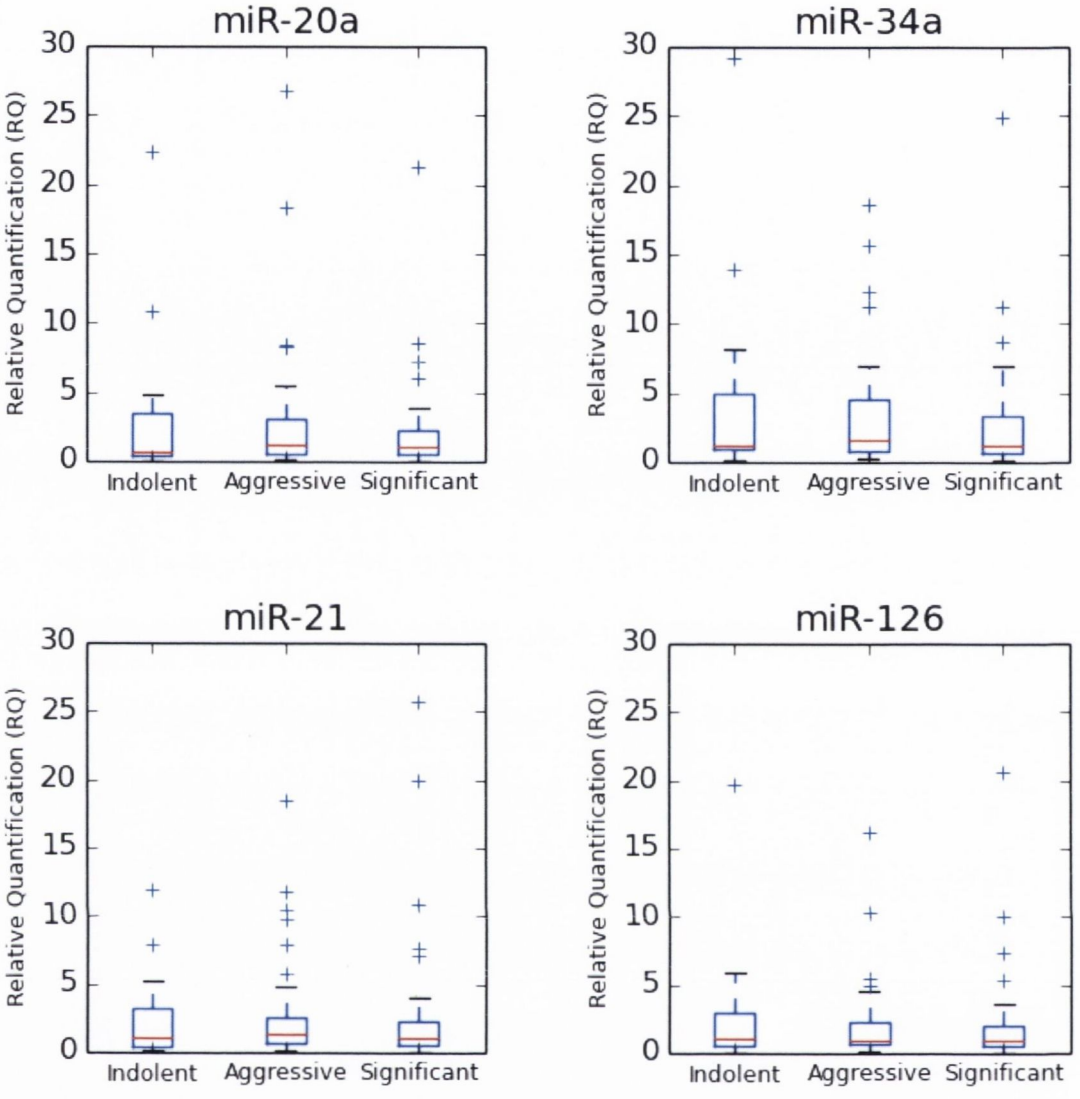

Figure 4.11 *Boxplots of relative quantification (RQ) of expression of miR-20a, miR-34a, miR-21 and miR-126 across the tissue cohorts.*

No statistically significant difference in expression was identified. + indicates outliers.

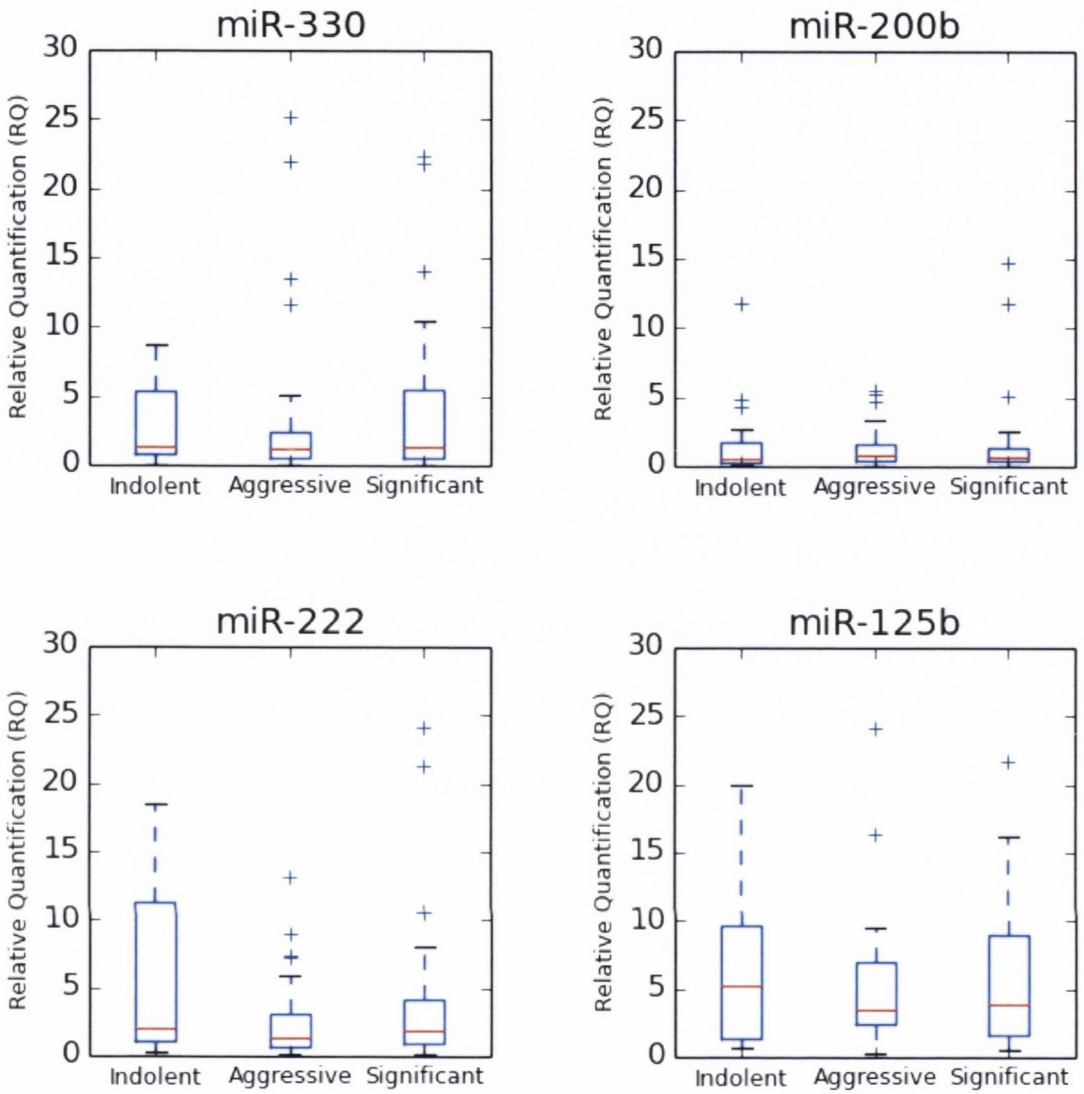

Figure 4.12 *Boxplots of relative quantification (RQ) of expression of miR-330. miR-200b, miR-222 and miR-125b across the tissue cohorts.*

No statistically significant difference in expression was identified. + indicates outliers.

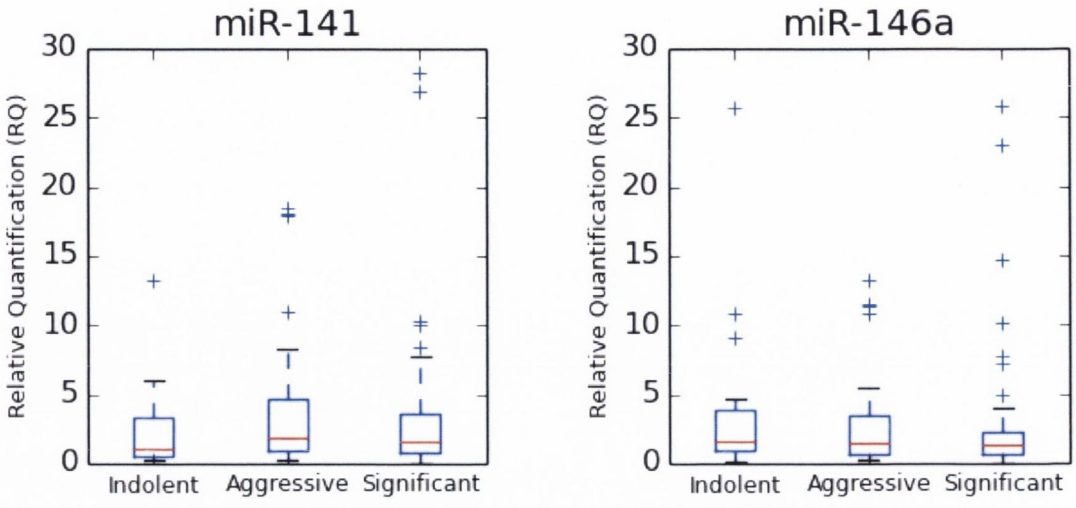

Figure 4.13 *Boxplots of relative quantification (RQ) of expression of miR-141 and miR-146a across the tissue cohorts.*

No statistically significant difference in expression was identified. + indicates outliers.

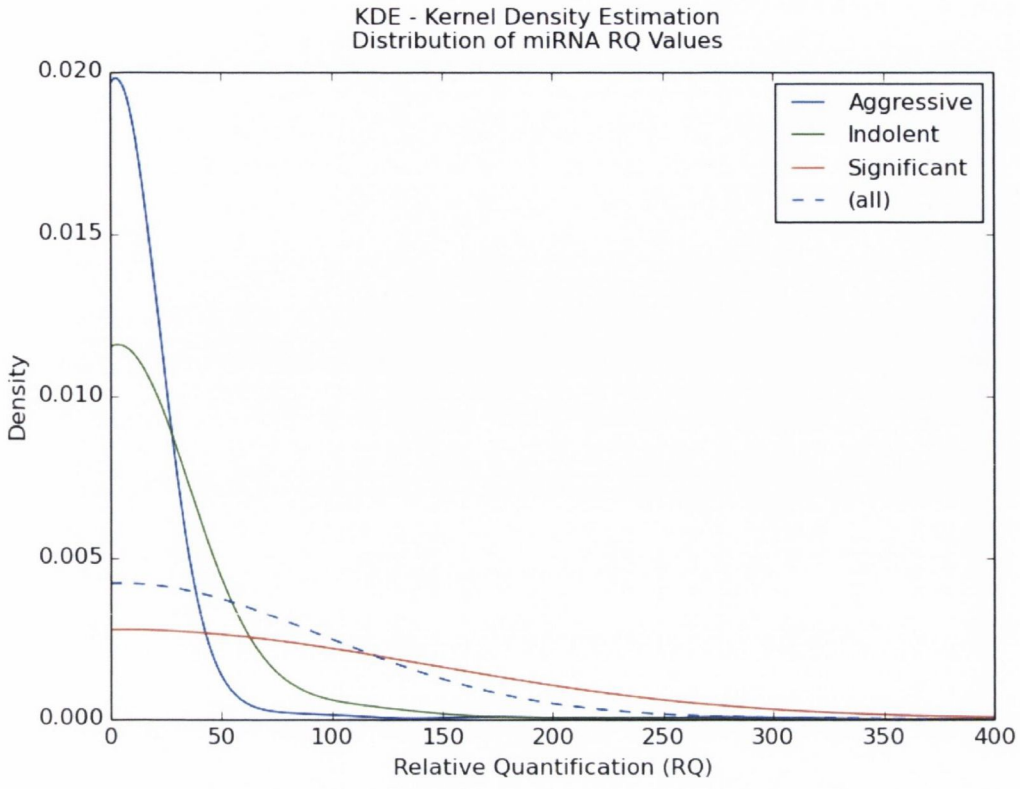

Figure 4.14 *Kernel density distribution of relative quantification (RQ) of miRNA expression.*

Kernel density estimation (KDE) was used to plot the probability density function of the relative quantification values of miRNA expression. The dashed line represents the data from the three cohorts represented as a whole. The blue curve represents the aggressive cohort, indicating that the probability of a large fold change in miRNA expression is highest in the aggressive group. The miRNA RQ patterns for each group are reciprocal to those observed for KDE of the gene expression RQ values, as would be expected.

4.2.5 Stem-associated miRNA Expression

The opportunity arose to perform an additional miRNA expression analysis in collaboration with PCRC colleagues using the Exiqon miRCURY Linked Nucleic Acid (LNA^{TM}) miRNA array technology. The same sample cohort was analysed and the samples were processed by Exiqon. The expression of four stem-associated miRNAs was examined; let-7a, let-7b, miR-145 and miR-143. An upper limit of C_q=37 (C_q : quantification cycle, the number of cycles required for the fluorescent signal to cross the threshold) was established whereby cycle threshold values of 37-40 were considered as absent expression. Samples were normalised to the average C_q of three endogenous control assays; let-7e, miR-342 and miR-92a.

Pairwise comparison was performed between groups in order to identify whether differential stem-associated miRNA patterns associate with a particular tumour classification; Indolent vs. Aggressive, Aggressive vs. Significant and Indolent vs. Significant. Two miRNAs exhibited statistically significant differential expression patterns. miR-143 (p=0.002) and let-7a (p=0.02) were identified as statistically significantly upregulated in the aggressive group when compared to indolent, while no statistically significant differences in miRNA expression were found when the significant group was compared to the aggressive group. miR-143 (p= 0.02) was once again, found to be statistically significantly upregulated in the significant group when compared to the indolent.

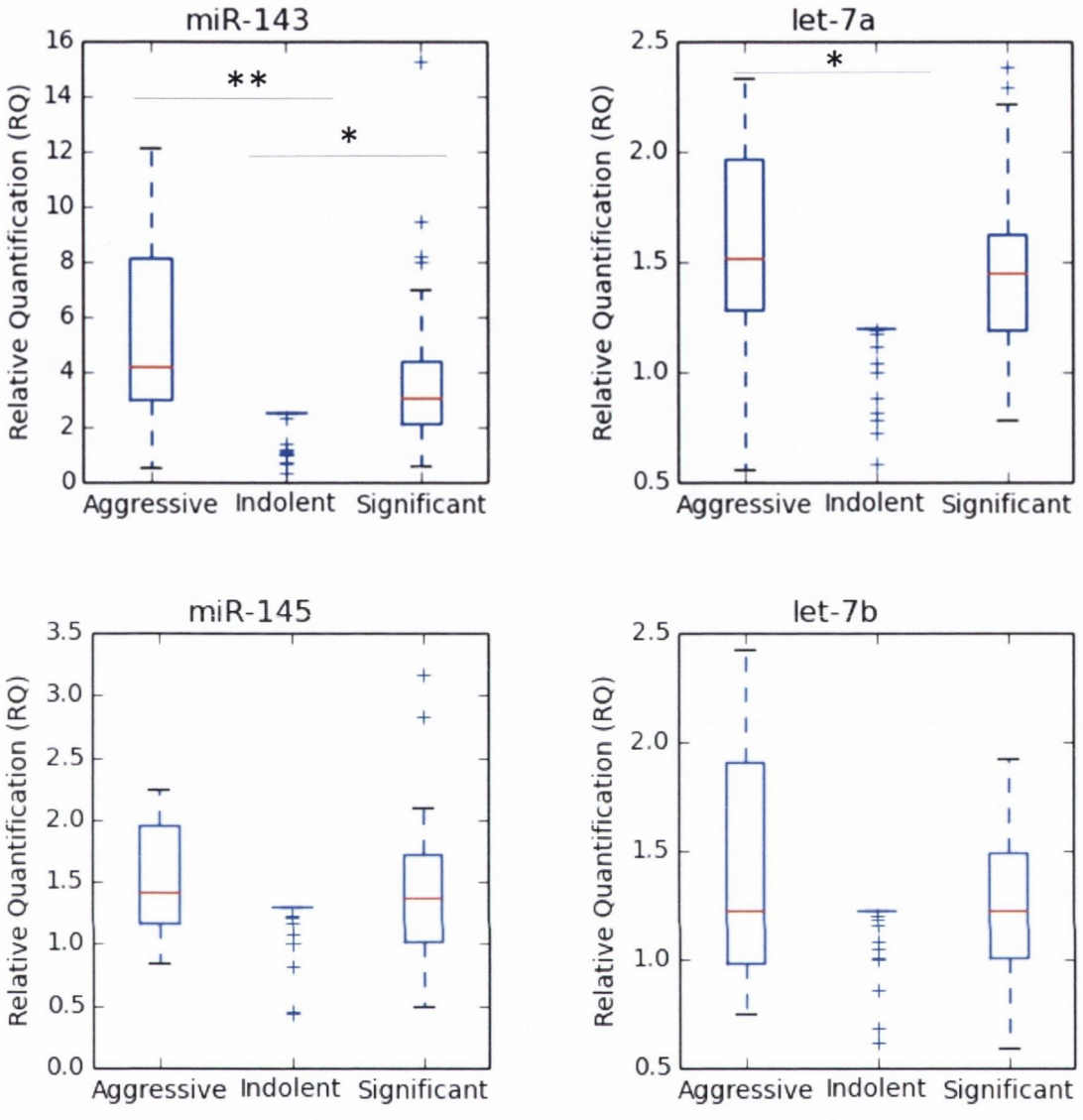

Figure 4.15 *Boxplots of relative quantification (RQ) of change in expression of miR-143, miR-145, let-7a and let-7b between aggressive, indolent and significant groups.*

miR-143 was found to be statistically significantly upregulated (p=0.002) in the aggressive group when compared to indolent. miR-143 was also found to be statistically significantly upregulated in the significant group when compared to the indolent group. While let-7a was also found to be statistically significantly upregulated in the aggressive group when compared to the indolent (p=0.02). No significant change in expression in let-7b and miR-145 was identified.

* indicates statistical significance ($p < 0.05$) between groups as identified by Mann-Whitney U test. + indicates outliers.

4.3 Discussion

Despite considerable advancements in the early detection of prostate cancer, the ability to differentiate aggressive from non-aggressive prostate tumours remains a clinical enigma (Barry *et al.,* 2001). The inadequacy of current predictors of prognosis, namely PSA measurement, Gleason score and clinical stage is the fundamental reason for no real improvement in the quality of life of patients despite the ability to detect this disease at a remarkably early stage (Huppi *et al.,* 2004). The heterogeneous nature of prostate cancer is postulated to contrive a complex molecular genetic expression profile, which has confounded many attempts to establish a robust biomolecular marker of aggressive disease. To this end, an informed genomic profiling of a well-defined clinical cohort was performed as part of a multi-institutional collaborative enterprise by the prostate cancer research consortium to molecularly characterise aggressive prostate cancer. The strength of this study in its entirety lies within the synergistic approach adopted by the PCRC, as each study component has focussed on the same sample set, ultimately delivering a comprehensive analysis of transcriptomic, epigenetic and proteomic patterns across the patient cohort. It is postulated that this thorough analysis will facilitate the molecular taxonomic subclassification of aggressive disease and advance the development of a non-invasive tumour-tracking liquid biopsy. However, a significant limitation at present which complicates the extrapolation of relevant conclusions from the data garnered by this study is the relative immaturity of the patient cohort. Currently, this study can act merely as a baseline, which will grow in importance as the cohort matures. It is hoped that transcriptomic data will become more relevant in the coming years as patients are monitored longitudinally and outcome measures are established. Over time, as a proportion of patients inevitably experience biochemical recurrence and disease progression; the PCRC bioresource will provide an invaluable source of retrospective data from which appropriate conclusions can be made. Thus, at present while the potential significance of this expression analysis can be explored, it is impossible to definitively state whether the differentially expressed targets are true biomolecular markers of intrinsically aggressive prostate carcinoma.

4.3.1 Differential Gene Expression Characterises Aggressive Disease within the PCRC Cohort

Examination of the expression profiles of the eleven genes within our panel identified statistically significant differential expression of six genes, all of which were found to be upregulated in the aggressive patient cohort when compared to both indolent and putatively significant disease; IGFBP3, SFRP4, CCNB1, FAM49B, AMACR, MUC1.

Insulin-like growth factors (IGFs) are powerful multifunctional mitogens which regulate proliferation, differentiation and cell apoptosis (Renehan *et al.,* 2004). IGFs circulate via interactions with IGF-binding proteins (IGFBPs), which modulate the activity of the IGF-signalling pathway (Kanety *et al.,* 1993). Insulin-like growth factors are abundant in the circulation and exert systemic, hormonal and local paracrine effects on cell behaviour (Renehan *et al.,* 2004). Within the circulation, the predominant IGF-binding protein is insulin-like growth factor binding protein 3 (IGFBP3), which associates with IGF-1. Inter-individual variability in the concentration of this peptide is considerable as its levels are dependent upon growth hormone, and can be affected by age, sex and nutritional status, which complicates its quantification within a population (Rajaram *et al.,* 1997). Early studies on prostate cancer risk have indicated that low serum levels of IGFBP3 directly increase the risk of advanced stage prostate cancer (Chan *et al.,* 2002). Furthermore, preoperative circulating levels of IGFBP3 have been demonstrated to be independent predictors of prostate cancer progression in multivariate models which include conventional clinicopathological variables such as PSA status and Gleason score (Shariat *et al.,* 2002). IGFBP3, conventionally considered as an antiproliferative peptide as a result of its ligand sequestration abilities, has also been implicated in the inhibition of the NF-κB pathway independent of its ability to sequester IGFs (Han *et al.,* 2011). Infact, IGF-independent mechanisms are believed to mediate much of the tumour-suppressive activities of IGFBP3 (Han *et al.,* 2011).

However, a recent study has demonstrated that high nuclear IGFBP3 staining in primary prostate tumours has statistically significant associations with prostate cancer recurrence (Seligson *et al.,* 2013). Immunohistochemistry was performed on tissue microarrays comprising 226 hormone naive patients treated with radical prostatectomy for localised prostate cancer. While a broad range of IGFBP3-staining was observed across all histologies examined, tumour was noted to exhibit both stronger nuclear and cytoplasmic staining than benign tissue. This high level of nuclear staining in particular was found to be a more accurate predictor of biochemical failure than preoperative PSA, clinical stage and tumour margin status. The elevated nuclear positivity for IGFBP3 is thought to be a repercussion of the presence of a mutation within a nuclear export sequence, which is postulated to isolate this peptide within the nucleus, ultimately negating its apoptotic-inducing capacity (Paharkova-Vatchkova *et al.,* 2010). Thus, post-translational modifications during prostate cancer progression are hypothesised to mediate nuclear sequestration and subsequent inactivation of IGFBP3. While these findings demonstrate a pathologic consequence of abundant IGFBP3, it is difficult to determine the significance of our observed IGFBP3 overexpression in the aggressive patient cohort. However, it is noteworthy that IGFBP3 is a direct target of miR-100, the expression of which has been demonstrated as

depleted in metastatic prostate carcinoma when compared to localised disease (Leite *et al.,* 2011). Infact, the loss of miR-100 is postulated to play a pivotal role in the development of bone metastasis in prostate cancer patients, potentially mediated by the activity of cancer stem cells as this miRNA also targets a variety of potent stem-associated genes; c-Myc, SOX2, and Klf4 (miRWalk database). Thus, IGFBP3 overexpression may occur merely as a consequence of progressive miRNA dysregulation. As previously mentioned, IGFBP3 concentration is influenced by exogenous factors including hormone levels and it is likely that these factors play an additional role in the variability of this peptide between individuals.

Secreted frizzled-related proteins (SFRP) are extracellular proteins which antagonise Wnt signalling through the sequestration of Wnt ligands (Bafico *et al.,* 1999). Wnt proteins are a highly-conserved, ligand family which mediate a variety of cellular processes including cell fate determination, proliferation and cell polarity (Horvath *et al.,* 2004). The Wnt/β-catenin pathway has been implicated in the development of multiple cancer types including breast and colon. Inactivation of this pathway results in the formation of a complex between glycogen synthase kinase 3β (GSK3β) and axin/adenomatous polyposis coli. This complex catalyses the phosphorylation and subsequent degradation of β-catenin. Conversely, constitutive activation of this pathway inhibits the activity of GSK3β which allows β-catenin to accumulate in the cytoplasm. The eventual translocation of β-catenin to the nucleus results in the expression of proliferation-associated genes such as cyclin D1 and c-myc, through the binding and activation of transcription factors (Miller, 2002). SFRP4 binds Wnt ligands to inactivate this pathway (Uren *et al.,* 2000). Epigenetic silencing of Wnt antagonists been implicated in the development of multiple cancer types including breast and colon, suggesting a potential role as tumour suppressors (Polakis *et al.,* 2000; Miller, 2002). However, gene expression profiling studies have demonstrated that secreted frizzled-related protein 4 (SFRP4) is more abundant in malignant prostate tissue than benign tissue. A study by Horvath *et al.,* (2004) has demonstrated that radical prostatectomy specimens routinely express high levels of cytoplasmic SFRP4 mRNA, while only a small proportion express membranous SFRP4. Furthermore, those patients with less than 20% of cells expressing membranous SFRP4 had a decreased progression-free survival time (median: 45 months), while those who had > 20% of cells expressing membranous SFRP4 had a significantly increased progression-free survival (median: 65 months, $p=0.002$). These results would appear to indicate that increased membranous expression of SFRP4 predicts a longer relapse-free survival in patients with localised prostate cancer, which suggests that the localisation of SFRP4 is critical to determining the pathogenic outcome of its dysregulated expression. There is much evidence to indicate that aberrant expression of SFRP4 is an innate characteristic of malignant prostate tissue, however the precise cause and

consequence of abundant SFRP4 transcript is unclear. A search of the validated miRNAs known to target SFRP4 on the miRWALK database identifies miR-31 as the sole small RNA regulator of this gene. Strikingly, a recent study by Lin *et al.,* (2013) has demonstrated the diminished expression of miR-31 in metastatic prostate cancer as a result of promoter hypermethylation (Lin *et al.,* 2013). Furthermore, the levels of miR-31 were found to inversely correlate with the aggressiveness of the disease. This study has demonstrated a novel role for miR-31 in a mutual feedback loop with the androgen receptor (AR); the AR is postulated to regulate the expression of miR-31, while miR-31 directly targets the AR at a conserved site within the coding region, which ultimately exerts a tumour-suppressive effect. Thus, the downregulation of miR-31 as a result of aberrant epigenetic modification may contribute to the progression of prostate cancer in an AR-mediated manner. Perhaps overexpression of SFRP4 in aggressive prostate cancer is merely a repercussion of miR-31 deficiency. Unfortunately, miR-31 was not a candidate within our miRNA panel and so further investigation would be required in order to establish a causal link between miR-31 depletion and SFRP4 overexpression in aggressive prostate neoplasms.

FAM49B (family with sequence similarity 49, member B) represents a relatively novel potential disease marker. This gene target was originally identified as a member of the systemic progression signature identified by Nakagawa *et al.,* (2010) however its pathological role in prostate cancer progression remains unclear. It has been identified as a target of the BACH1 (BTB and CNC homology 1) transcription factor (Warnatz *et al.,* 2011). This heme-binding transcription factor has been implicated in the physiological regulation of oxidative stress through the repression of its predominant target gene HMOX1 (Sun *et al.,* 2002). BACH1 has recently been found to repress the expression of an additional 59 genes (Warnatz *et al.,* 2011). Following BACH1 knockdown, target mRNA transcripts were found to be highly abundant, indicating the strength of BACH1-mediated repression. The genes targeted by this transcription factor are implicated in a variety of diverse pathways involved in the maintenance of cellular homeostasis including heme degradation, redox regulation, proliferation and apoptosis. Interestingly, the BACH1 transcription factor has been implicated in the development of carcinogenesis via miRNA-mediated downregulation. For example, overexpression of miR-155 resulting in the silencing of BACH1 has been demonstrated in B-cell lymphomas (Costinean *et al.,* 2006). Thus, the observed overexpression of FAM49B may represent the consequence of a regulatory 'domino-effect' whereby miRNA dysregulation may result in the inhibition of BACH1 and concomitant constitutive expression of its targets; including FAM49B.

Cyclin B1 (CCNB1) functions in the regulation of G(2)-M phase transition in mitosis. Overexpression of this protein has been demonstrated in multiple cancer types and its

dysregulation is thought to be an early event in the development of neoplasia (Pines *et al.,* 2006). Abundant levels of CCNB1 have also been implicated in the poor clinical outcome of non small cell lung cancer and head and neck squamous cell carcinoma (Soria *et al.,* 2000; Hassan *et al.,* 2002). A study by La-Tulippe *et al.,* (2002) has examined the genomic phenotype of primary and metastatic prostate cancers by performing a genome-wide expression analysis. This study identified a panel of significantly differentially expressed genes between the two cohorts. CCNB1 and CCNB2 were identified as two of the most significantly enriched genes in metastatic tumours. Thus, it is not entirely surprising that, cyclin B1 has emerged as overexpressed in our aggressive patient cohort, given its critical role in cell cycle progression. A hallmark of the malignant state is indiscriminate cell division and as previous data demonstrates aberrant expression of cyclins and the resultant uncontrolled activity of cyclin-dependent kinases is largely responsible for this.

α-Methyl CoA Racemase (AMACR) is a peroxisomal and mitochondrial enzyme which plays a critical role in the β-oxidation of branched-chain fatty acids. This enzyme catalyses the conversion of (2*R*)-methyl-branched-chain fatty acyl-CoAs to their (*S*)-stereoisomers (Ferdinandusse *et al.,* 2000). Gene expression studies have demonstrated that AMACR is abundantly expressed in malignant prostate tissue relative to benign tissue (Rubin *et al.,* 2002). Indeed, a meta-analysis of four microarray data sets has identified AMACR as one of the most consistently overexpressed genes in prostate cancer (Rhodes *et al.,* 2002). As a result, the expression of AMACR is often quantified in atypical needle biopsy specimens to definitively diagnose malignancy (Rubin *et al.,* 2002). While the aberrant expression of AMACR has proven clinically useful as an adjuvant tool in the diagnosis of prostate cancer, little has been reported in terms of an association between elevated AMACR expression and prostate cancer aggression. A study by Bismar *et al.,* (2006) has identified a 12-gene model for the prediction of prostate cancer progression. This study employed a similar data integration approach, combining the results of previous high-throughput proteomic and expression array analysis studies to identify a gene target panel. This approach yielded a panel of 50 genes with concordant overexpression of transcriptional and subsequent protein products. A subsequent linear discriminant analysis refined this panel to 12 genes whose dysregulated expression was hypothesised to accurately predict prostate cancer progression. Two candidate genes within this panel were AMACR and MUC1 (a gene which has previously been significantly associate d with disease recurrence (Lapointe *et al.,* 2004); both of which have emerged as statistically significantly overexpressed in putatively aggressive prostate cancer in the current study. To determine whether transcriptional levels of these 12 genes could accurately identify those prostate cancers at the greatest risk of progression following radical prostatectomy, a previously published independent data set of

79 tumours was analysed (Glinsky *et al.,* 2004). Expression array clustering and Kaplan-Meier analysis identified a significant separation between two major sample clusters with regards to PSA failure. Thus, this 12-gene model was found accurately to discriminate those patients with localised prostate cancer, who were at the highest risk of biochemical recurrence following initial treatment.

These results are highly suggestive of the potential clinical utility of AMACR as a predictive marker for long-term clinical outcome; however a study by Rubin *et al.,* (2002) would appear to contradict these findings and provides an admonitory reminder of the perils associated with implementing surrogate endpoints such as PSA failure in studies of this kind. This study specifically explored the adequacy of AMACR as a biomarker for aggressive disease. Expression of this protein was quantified by immunohistochemistry in two cohorts of men with localised prostate cancer; one consisted of 204 patients treated with radical prostatectomy while the other comprised 188 patients managed with surveillance. A regression tree method was utilised to define the optimal AMACR protein expression cutpoints which best differentiated prostate cancer outcome in the individual cohorts. The effect of the AMACR cutpoints on clinical outcome was then examined. The results of this analysis indicated that lower expression of this protein was significantly associated with worse clinical outcome. Furthermore, among those patients with low AMACR expression and high Gleason score, the risk of prostate cancer specific-mortality was 18-fold higher indicating a joint association between this putative biomarker and the current clinical measure of Gleason score.

Similar to the study by Bismar *et al.,* (2006), the endpoints implemented in this study were PSA failure and time to prostate cancer-related death in the active surveillance cohort. Interestingly, the authors noted that the AMACR cutpoints differed depending on whether they were derived from PSA-failure or prostate cancer-specific death. While there is a significant degree of contention surrounding the lack of standardised methods for the interpretation of immunohistochemistry, the discrepancy within this study and the lack of concordance between studies is likely a result of the use of inappropriate surrogate endpoints to mark disease progression. Most biomarker studies have utilised surrogate markers of preclinical recurrence in order to reduce the duration and size of a study, however it has been postulated that studies based upon surrogate end points lack the credibility of those with a 'true' clinical end point. PSA failure following primary treatment has long been recognised as a surrogate marker for systemic progression and ultimately prostate cancer-related mortality (Stamey *et al.,* 1999). However, the lack of agreement between studies suggests that PSA failure may not be an optimal end point. While our results demonstrate that AMACR is significantly differentially expressed in the putatively

aggressive patient cohort, we are unable to presently establish any association between AMACR overexpression and legitimately aggressive disease; however previous studies would suggest that the implementation of surrogate clinical parameters to define aggressive disease is not optimal for the development of predictive biomarkers.

4.3.2 Confounding Factors in the Quantification of miRNA Expression

Extensive miRNA dysregulation is a prevailing feature of human cancer. The discovery that miRNAs are involved in virtually all physiological and pathological processes has spawned intense interest in the identification of disease-specific miRNA expression signatures. As a result, many studies have reported the identification of expression signatures which can accurately differentiate benign and carcinoma samples. Furthermore, there is evidence to indicate that dysregulated miRNA expression patterns become more chaotic and discordant as malignancy progresses (Tong *et al.,* 2009). Thus, one would expect, given the widely reported pathologic role of miRNA deregulation in prostate cancer, that our results would support these findings however our analysis has identified no significant differential miRNA expression patterns across the three patient cohorts. These results are somewhat surprising considering the concomitant identification of differential gene expression patterns in these samples. The absence of differential miRNA expression is likely a result of a number of challenges associated with the accurate detection of temporal miRNA expression changes.

Previous studies have indicated that miRNAs are exquisitely fine yet powerful regulators of gene expression and as a consequence, subtle changes in miRNA expression are sufficient to exert profound effects upon the regulation of genes within a biological network (Heneghan *et al.,* 2010). Thus, the varying magnitude of these transitory changes in miRNA expression during tumorigenesis combined with the naturally occurring noise in biological samples make them relatively challenging to accurately quantify. Despite the development of increasingly sensitive assays for the detection of miRNAs in the intervening years since their discovery, one must be cognisant to the need for further advancements in this area. While high-throughput RT-PCR arrays are the most common method associated with the detection of miRNAs, there are a number of disadvantages inherent in this technique. Firstly, batch-to-batch variability in the manufacturing of each card can reduce sensitivity and specificity of the hybridised probe (Schmittgen *et al.,* 2008). Furthermore, difficulty associated with accurately transferring liquid into 384-well cards has been postulated to decrease the accuracy of this technique. These constraints, if at play are likely to only marginally affect the accurate quantification of miRNA expression changes and it is highly probable that the complex interaction between tumour cells and the local microenvironment is a determinant in the legitimate measure of expressional changes.

The concept that local microenvironmental signals may influence and perhaps promote expansion of the malignant cell population is by no means a recent idea and the role of various cell types within this complex interplay is slowly being elucidated (Reedy, 1975). An early study by Schor and colleagues, demonstrated that fibroblasts surrounding malignant epithelium were radically dissimilar from normal stroma and that these fundamental differences were associated with disease progression (Schor *et al.,* 1987). Furthermore, this disparity in cellular identity was observed in only a small subset of resident fibroblasts (Schor *et al.,* 1988). These observations have founded intense research efforts to delineate the role of fibroblasts, myofibroblasts and reactive stroma in neoplastic progression. In particular, a number of important studies have examined this cacophonous cellular hierarchy in prostate cancer. Yanagisawa *et al.,* (2007) have demonstrated that grading of the reactive stromal phenotype is an independent predictor of disease recurrence. This study demonstrated the utility of this stromal grading system in determining biochemical recurrence-free survival in preoperative needle biopsy specimens, indicating that the recognition of stromogenic carcinoma can inform clinical outcomes. These results provide compelling evidence implicating the surrounding stroma in the regulation of tumorigenesis, however this situation appears chaotic and there are many cellular interactions which remain to be understood. Nonetheless, the undeniable relationship between tumour cells and the spatial microenvironment does provide a basis to postulate that perhaps the most cogent and aggressive disease-specific transcriptomic alterations are occurring in the tumour stroma. Our expression analysis was fuelled by highly-enriched epithelial tissue derived from the most predominant tumour lesion within the specimen. In order to rule out the possibility of tumour-stromal interactions sustaining and indeed driving tumour progression particularly in aggressive carcinoma, it would be necessary to study both the primary tumour lesion and the surrounding stroma. Thus, it is pertinent to hypothesise that disparate clinical outcomes in prostate cancer may be a result of heterogeneous stromogenic tumour phenotypes.

4.3.3 Stem-associated miRNAs are Preferentially Enriched in the Aggressive Patient Cohort

As previously discussed, cancer stem cells have been implicated in therapeutic resistance, disease progression, and systemic metastasis and as a result of these associations, hold significant clinical relevance. The discovery that miRNA expression patterns in embryonic stem cells closely parallel those of cancer cells has led to the postulation that the self-renewal and proliferative properties inherent in cancer stem cells are regulated by a diverse network of miRNAs (Yu *et al.,* 2007). As the malignant phenotype is considered a consequence of the disruption of intrinsic cancer stem cell properties, we hypothesised that differential stem-associated miRNA patterns may characterise aggressive prostate cancer.

miR-143 has been identified as the most significantly enriched miRNA during differentiation of mouse embryonic stem cells into multipotent cardiac progenitors. Both miR-143 and miR-145 are highly conserved miRNAs, located on mouse chromosome 18 (Cordes *et al.,* 2009). These miRNAs are believed to be transcribed from a bicistronic unit, suggesting that their expression is modulated by shared regulatory elements. Furthermore, the pluripotency factors OCT4, SOX2 and Klf4 have been identified as direct targets of miR-145 and increased expression of this miRNA inhibits the self-renewal of human embryonic stem cells and induces lineage-restricted differentiation (Xu *et al.,* 2009). Thus, there is much evidence to substantiate a link between these miRNAs and the regulation of embryonic stem cell characteristics. It has been postulated that many of the properties inherent in embryonic and normal epithelial stem cells closely parallel that of their malignant counterpart (Zhang *et al.,* 2008). Furthermore, both miR-143 and miR-145 are known to be dysregulated in various cancer cell lines and primary human tumour types including lung and colon (Calin *et al.,* 2006). Thus, it may be hypothesised that miR-143 and miR-145 are implicated in the progression of prostate cancer through the regulation of cancer stem cell function.

Expression analysis found miR-143 to be significantly differentially expressed across our three patient cohorts. Pairwise analysis revealed that miR-143 was significantly overexpressed in the aggressive patient group when compared to the indolent. In addition, it was found to be overexpressed in the significant group when compared to the indolent group. A recent study by Fan *et al.,* (2013) has utilised serum-free medium culture to generate a model of prostate cancer stem cells through the derivation of PC-3 spheres. These spheres were shown to significantly overexpress the embryonic stem cell-associated markers OCT4/POU5F1, SOX2 and NANOG when compared to adherent cells. Furthermore, progressive downregulation of these stem markers was shown to accompany the reintroduction of PC-3 spheres to adherent culture. Examination of the miRNA expression profiles of both adherent cells and putative cancer stem cell-containing spheres revealed that miR-143 was downregulated 8.4-fold in sphere cells when compared to their adherent counterpart. Furthermore, expression of miR-143 was found to progressively increase as sphere cells differentiate upon reintroduction to adherent culture. These findings suggest a role for miR-143 in the differentiation of cancer stem cells. Interestingly, transwell assays demonstrated that more adherent PC-3 cells penetrated the gel membrane than sphere cells; however sphere cells that were digested and reintroduced to adherent culture exhibited drastically increased migration capability. These findings indicate that prostate cancer stem cells may possess a limited invasive potential but generate highly aggressive progenitor cells. Contrary to previous findings, Fan *et al.,* (2013) reported that the inhibition of miR-143 in adherent PC-3 cells strongly repressed the invasive potential of prostate

cancer cells *in vitro* indicating a potential role for the overexpression of miR-143 in metastasis. Furthermore, *in vivo* studies demonstrated that mice injected with miR-143-inhibited PC-3-M cells developed fewer systemic metastases and fewer macroscopic nodes in the liver than those injected with miRNA-negative control-transfected cells. These findings provide compelling evidence implicating miR-143 in the development of distant metastases. FNDC3B (fibronectin type III domain containing 3B) has been identified as a direct target of miR-143. This gene functions in the regulation of cell motility and has been reported as downregulated in malignant cells with high metastatic potential (Urtreger *et al.,* 2006). miR-143 overexpression has previously been associated with the development of metastasis in hepatocarcinoma through the repression of FNDC3B (Zhang *et al.,* 2009). Thus, Fan *et al.,* (2013) hypothesised that miR-143 dysregulation may be associated with metastasis through the modulation of FNDC3B expression. Furthermore, a recent study by Rane *et al.,* (2015) has identified miR-143 as one of ten miRNAs upregulated in castrate-resistant prostate cancer stem-like cells.

Taken together, these findings would appear to suggest that the upregulation of miR-143 in prostate carcinoma could result in the deranged differentiation of cancer stem cells to yield a distinctive subset of phenotypically aggressive cells, which possess an enhanced metastatic capacity. The observed overexpression of miR-143 in the aggressive patient group is consistent with this hypothesis. While this postulation provides an attractive mechanism to reconcile the overexpression of miR-143 with the aggressive tumour subtype, the extent to which the stem population is truly affected by these miRNA changes remains unclear and more likely it will be necessary to isolate this cellular population and perform an exhaustive genetic analysis, in order to truly appreciate the miRNA expression patterns which guide cancer stem cell function.

The let-7 family of miRNAs are absent in embryonic stem cells and this finding has been mirrored in expression analyses which have identified the coordinate downregulation of the let-7 family in human cancers including breast, lung and ovarian. Breast cancer stem cells were the first putative cancer stem cells to be identified in solid tumours and as such the miRNA expression patterns which regulate this cellular population are better understood. A study by Yu *et al.,* (2007) has demonstrated that a breast cancer stem cell-enriched population with the antigenic phenotype $CD44^{+}CD24^{-/lo}$ expressed much lower levels of let-7 than differentiated parental cells. Lentiviral-mediated overexpression of let-7a in putative breast cancer stem cells has been shown to inhibit cell proliferation, mammosphere formation, tumour formation and metastasis in NOD/SCID mice. In contrast, antagonisation of let-7 by antisense oligonucletoides enhanced the *in vitro* self-renewal of differentiated progenitor cells. Furthermore, HMGA2 and H-RAS were identified as direct targets which

partially mediate the downstream effects of let-7 (Yu *et al.,* 2007). These findings have been replicated in a study by Liu *et al.,* (2011), which identified putative prostate cancer stem cells expressing high levels of the surface markers CD44, CD133 and α2β1. These tumour-initiating stem-like cells were also found to highly underexpress let-7b (Liu *et al.,* 2011). Thus, in support of the cancer stem cell hypothesis, many studies suggest that the epigenetic downregulation of let-7 in cancer stem/multipotent progenitor cells is a common event and results in the upregulation of oncofetal genes including HMGA2, lin28, Ras and Myc, which concomitantly enhances stem activity and perpetuates tumorigenesis.

Our results have demonstrated the statistically significant upregulation of let-7a in the aggressive patient cohort compared to the indolent. At this juncture, it is worthy to note that there is a significant degree of contention surrounding the expression levels of let-7 in human cancers. Let-7 is a widely accepted tumour suppressor miRNA, whose loss in malignant cells is postulated to predict poor survival (Coppola *et al.,* 2010). However, upregulation of certain let-7 family members, in particular let-7a has been demonstrated in multiple cancer types. For example, Tong *et al.,* (2009) performed an expression profiling of 40 prostatectomy specimens ranging from stage T2a/b to early and late-stage relapse cases. This study demonstrated the statistically significant upregulation of let-7a in malignant prostate tissues compared to normal tissue. Furthermore, dysregulation of this miRNA was found to be significantly associated with late-stage relapse disease. These findings have been replicated in a similar study by Garzon *et al.,* (2008), which examined miRNA expression in untreated AML patients. Let-7a was identified as statistically significantly upregulated in AML patients. In addition, a significant association was observed between let-7a upregulation and high-grade disease. Thus, these studies provide compelling evidence that let-7 functions in a cell-specific manner and its dysregulated expression may have varying pathologic outcomes depending upon cell context. A recent study by Rane *et al.,* (2015) has provided further evidence in support of this hypothesis. miRNA expression profiles were generated from prostate epithelial subpopulations derived from benign prostatic hyperplasia (BPH) specimens, Gleason 7 treatment-naive radical prostatectomy specimens and castrate-resistant prostate carcinoma. Principal component analysis demonstrated that miRNA expression was clustered by epithelial cell phenotype irrespective of pathologic status. These findings indicate that fractionation of prostate tumours is pivotal in identifying accurate expressional differences. Furthermore, the downregulation of let-7 was identified as a signature of conserved stem cells, however let-7 was found to be upregulated in a purified population of castrate-resistant prostate cancer stem-like cells. This data strongly suggests that the failure to resolve cell subtype-specific miRNA expression differences is the

predominant reason behind the marked heterogeneity and subsequent lack of concordance among expression profiling studies.

Despite the clinical significance of cancer stem cells, little is known regarding the molecular regulation of this putatively rare subpopulation. Dysregulated miRNA expression is strongly associated with tumour initiation and progression and as a result it has been hypothesised that miRNAs may exert their pathologic effect through the subversion of stem cell properties. Our results indicate that the deregulation of miRNAs, which function in the maintenance of cancer stem cell identity, may induce the disorganised differentiation of stem-like cells yielding highly aggressive, tumorigenic pools of distinct multipotent progenitors. However, in this instance one must be conscious of the broad spectrum of heterogeneity symptomatic of neoplastic malignancies. In recent years, a new appreciation has emerged for the heterogeneous cellular composition of human tumours; an interconnected milieu of dividing, differentiating and dying cells, infiltrating inflammatory components and of course, the supporting stroma (Williams *et al.,* 2013). The underlying genetic diversity means that tumours do not have an isolated driver mutation that can be directly targeted or quantified (Stephens *et al.,* 2012). In order to further understand tumour pathobiology, particularly the elucidation of cancer stem cell mechanisms, it will be necessary to study discrete tumour cell subpopulations in greater detail. The profound complexity of human tumour composition will not be overcome by the implementation of overly simplistic paradigms to characterise tumour subtypes. Critical to improving our understanding, is the availability of tumour models, which accurately recapitulate the functional and phenotypic heterogeneity of human tumours, in addition to the identification of a robust method of cancer stem cell demarcation.

4.4 Conclusion

A significantly reported clinical plight is the identification of patients with seemingly low-risk organ-confined prostate cancer who theoretically should fare well, but do not. This paradox epitomises the palpable and ever-growing dilemma frustrating the effective clinical management of prostate carcinoma. Efficient prognostication of this hugely prevalent malignancy is hindered by overtreatment of the indolent form and missed early intervention of the as yet uncharacterised inherently aggressive subtype. Upon its inauguration, the fundamental research goal of the PCRC was to dissolve these clinical issues by characterising the pathobiology of aggressive prostate cancer to deliver a robust biomolecular marker of this subtype, which could be translated into a clinically feasible, informative 'liquid biopsy'.

However, biomarker studies of this kind are complicated from the outset by the question they aim to answer. How is aggressive disease accurately quantified? The defined clinical cohort

examined in this study was subclassified based on a modified version of Epstein's Criteria (Epstein *et al.,* 1994). All aggressive disease cases were predicated on substantial evidence of extracapsular extension upon pathological analysis of radical prostatectomy specimens, essentially characterising disease based upon its invasive propensity. As the relatively immature cohort establishes itself in the coming years, it will become apparent whether this approach is feasible; however a more pressing and perhaps pertinent issue is the implementation of relevant disease end points to determine candidate biomarker:disease outcome associations. There are significant problematic issues associated with defining and implementing surrogate markers in aggressive prostate carcinoma (Gomella *et al.,* 2014). The protracted natural history of this malignancy complicates the implementation of overall survival as a feasible endpoint. Additional confounding factors include the ambiguity surrounding post-therapeutic changes in PSA levels and the lack of standardised methods to define progression-free survival and time to progression (Gomella *et al.,* 2014). A sobering reminder that surrogate endpoints including PSA recurrence as a marker for cause-specific mortality may not provide the optimal method to define biomarker:disease outcome associations. It is noteworthy, that this may prove challenging in the coming years as data garnered by this study is interpreted.

As previously alluded to, is it not yet possible to definitively state whether the differential miRNA/gene expression patterns identified in this study are associated with disease outcome, however this transcriptomic analysis has raised some pertinent issues regarding the effective profiling of human biological specimens. The host stromal microenvironment has been hypothesised to stimulate the development and rate of human tumorigenesis; however the reactive stroma in prostate cancer remains relatively undefined (Tuxhorn *et al.,* 2002). Several studies have substantiated the involvement of the stromogenic phenotype in prostate carcinoma development and as a result it is becoming increasingly important to characterise the reactive stroma to establish key regulators and identify the mechanisms by which the extracellular matrix promotes carcinogenesis (Tuxhorn *et al.,* 2001; Tuxhorn *et al.,* 2002). Thus, the exhaustive characterisation of the aggressive prostate cancer subtype will likely require the complete delineation of the role of stromal reaction in tumour progression.

Functionally plastic, cancer stem cells possess the exclusive ability to regenerate tumours (Lobo *et al.,* 2007). The corruption of genes and miRNAs involved in the regulation of the intrinsic capabilities of this cellular population almost conclusively contributes to and exacerbates tumorigenesis, thus a complete understanding of this new paradigm of oncogenesis will provide a deeper insight into the ontogeny of human prostate tumours. Our results indicate that the gross dysregulation of cancer stem cell characteristics through the subversion of miRNA expression patterns may be associated with aggressive prostate

carcinoma. However, while the cancer stem cell phenotype is increasingly being corroborated as a signature of aggressive prostatic carcinogenesis it is unlikely that the molecular mechanisms governing cancer stem cell function can be truly elucidated without isolating this population from the bulk tumour. Thus, the demarcation and comprehensive characterisation of this cellular subpopulace is postulated to be of paramount clinical importance to understanding the pathobiology of aggressive prostate carcinoma.

Isolation and Characterisation of Prostate Cancer Stem Cells

Chapter 5

Chapter 5. Isolation and Characterisation of Prostate Cancer Stem Cells

5.1 Introduction

5.1.1 Cancer Stem Cells

Stem cells have long been known to occur in somatic tissues, which undergo rapid regeneration including bone marrow and the skin (Dexter *et al.,* 1977). However, in recent years the presence of stem cells has been recognised in more quiescent tissues, such as the prostate. Infact, the maintenance of prostate gland structure and function is dependent upon a repository of multipotent and stromal stem cells residing within the epithelial compartment (Foster *et al.,* 2002). Stem cells are characterised by their ability to potentiate their own proliferation through self-renewal, and to generate cells of multiple lineages. Thus, stem cells have the capacity for unlimited growth and can give rise to further stem cell progeny or cells with a more limited proliferative index known as transit-amplifying cells (Hall and Watt, 1989). Cells derived from stem cells are organised in a hierarchical manner, whereby self-renewing stem cells are situated at the pinnacle of the hierarchy.

The hierarchical organisation of morphologically heterogeneous cell types in normal tissue was first demonstrated in seminal work by Barrandon and Green (Barrandon and Green, 1987). This apparent heterogeneity in not only morphology but also functional expression patterns is thought to echo the varying developmental and maturation stages of normal stem and progenitor cells. This study demonstrated the relationship between colony-forming potential and stem capacity through culturing primary human keratinocytes in isolation. These cells were shown to generate a range of clonal morphologies with varying proliferative capacities, which they termed holoclones (*holo* = entire), meroclones (*mero* = partial) and paraclones (*para* = beyond). Holoclones were found to be capable of extensive self-renewal, while meroclones possessed limited proliferative ability and so were postulated to contain a dichotomy of proliferating and terminal cells. Paraclones were found to be incapable of further growth. These phenotypically plastic colonies are believed to derive respectively from stem, early and late-stage transit-amplifying cells. This unique pattern of hierarchical colony formation has been demonstrated in multiple immortalised cell lines and has since become a surrogate assay for the identification and characterisation of normal stem cells.

The observation that most human tumours are also heterogeneous in their histological composition has led many to posit that tumour development closely resembles normal tissue homeostasis (Dexter *et al.,* 1978; Sell *et al.,* 1994). Many of the properties inherent in stem-cells are highly relevant to human cancer (Reya *et al.,* 2001). Indeed, the 'cancer stem cell (CSC) hypothesis' is predicated on the basis that extensive tumour cell heterogeneity occurs as a direct result of the subversion of stem cell properties. The CSC hypothesis states that a

biologically distinct, rare subset of cells derived from the mutation of normal stem cells and their progenitors has the capacity to perpetuate the continued expansion of malignant cells. In support of this hypothesis, stem-like cells capable of recapitulating the complexity of human tumours have been identified not only in haematological malignancies but also in a variety of solid tumour types including; breast (Al-Hajj *et al.,* 2003), glioma (Singh *et al.,* 2004), colon (O' Brien *et al.,* 2007), and pancreatic cancer (Li *et al.,* 2007).

At present, there exists a significant degree of uncertainty concerning the study of prostate cancer stem cells. While it has been postulated that many cancers are initiated and perpetuated by stem-like cells, the extent to which tumour aggressiveness is influenced by cancer stem cells remains to be defined. As explored in section 1.8.2, the precise origin of normal prostate stem cells is incompletely understood. There is a large body of evidence to support both a basal and luminal phenotype for normal prostate stem cells. Furthermore, there is a great deal of debate on the prostate cancer cell of origin as it has been demonstrated that both basal and luminal cells can initiate tumour formation (Taylor *et al.,* 2012; Lu *et al.,* 2013). The ontogeny of prostate cancer stem cells is also poorly understood as it remains to be determined whether this subpopulation arises as a result of malignant transformation of normal stem cells or whether differentiated cells gain mutations, which concomitantly result in a reinitiation of stem characteristics. It has been postulated that cancer stem cells have the propensity to regenerate a tumour following otherwise successful primary treatment. They have also been implicated in the metastatic dissemination of cancer (Jordan *et al.,* 2006). For these reasons, there is a clinical requisite to characterise this cellular populace in greater detail. However, the establishment of cancer stem cell-targeting therapeutics is reliant upon the development of unequivocal methods of cancer stem cell isolation. This chapter will focus on the implementation of a number of varied methods of prostate cancer stem cell isolation and explore the advantages and limitations of these techniques. The fundamental issues we sought to address are whether immortalised prostate cancer cell lines retain the hierarchical cellular framework observed in normal epithelial cells and primary tumours, and whether the gene expression repertoires of differentially-derived putative stem cells reflect their developmental origin.

5.1.2 Identification of Prostate Cancer Stem Cells through Surface Marker Expression

For multiple tissue types, specific marker panels have been constructed, which are believed to define the stem cell population. As cancer stem cells are hypothesised to closely resemble normal stem cells in their biological behaviour, putative cancer stem cell fractions can be isolated on the basis of similar antigenic profiles as their normal counterparts. This technique has been demonstrated in multiple tumour types.

The quantification of specific cluster of differentiation (CD) surface markers has previously been employed to isolate both normal and cancer stem cells (Greve *et al.,* 2012). CD44 is a hyaluronan-binding cell surface glycoprotein with a number of critical roles in signalling, migration and homing (Visvader *et al.,* 2008). Expression of this adhesion molecule is routinely used to purify prospective cancer stem cell populations by fluorescent-activated cell sorting (FACS). Patrawala *et al.,* (2006) demonstrated that a purified $CD44^+$ population from multiple cultured prostate cancer lines and xenograft tumours displayed a high proliferative and clonogenic capacity. Furthermore, this population was found to be more tumorigenic than its negative counterpart upon transplantation into NOD/SCID mice. However, it was noted that this $CD44^+$ population was considerably heterogeneous comprising both early stem cells and more differentiated progenitors. Thus, this marker is generally used in combination with various other markers when identifying putative prostate cancer stem cells. The utility of integrins has previously been demonstrated in the identification of stem cells within the skin and testis (Li *et al.,* 1998; Shinohara *et al.,* 1999). These cell surface proteins are responsible for mediating the attachment of cells to extracellular matrix (ECM) proteins on the basement membrane. Collins *et al.,* (2005) were the first to report the identification of putative prostate cancer stem cells based on high surface expression of CD44 and integrin$\alpha_2\beta_1$ in conjunction with CD133 in primary and metastatic tumours. CD133 is a five-transmembrane domain glycoprotein, which has been implicated in the organisation of plasma membrane topology (Visvader *et al.,* 2008). This study demonstrated that only 0.1% of the cells within a tumour were triple positive for the CD44/integrin$\alpha_2\beta_1$/CD133 antigenic phenotype, which supports the idea that only a small fraction of tumour cells possess stem properties. Furthermore, this cellular fraction was found to possess the ability to self-renew and proliferate extensively *in vitro*. In addition, CD133-derived cultures displayed tumorigenic properties when replated in methylcellulose.

Studies have shown that the level of PSA expression in prostate tumours is directly proportional to the degree of differentiation (Qin *et al.,* 2012). Indeed, the existence of two populations, PSA^+ and $PSA^{-/lo}$, has been demonstrated in patient tumours. It has been shown that cells expressing little or no PSA are enriched in high-grade tumours and this $PSA^{-/lo}$ phenotype is generally associated with a poor prognosis due to the presence of worse clinical features, including metastasis and recurrence. Qin *et al.,* (2012) have demonstrated that this population of cells isolated from the LNCaP cell line, possess a number of stem-associated characteristics. In particular, they possess the ability to self-renew, a chief characteristic of both normal and cancer stem cells. They are also highly clonogenic and androgen refractory. The distinction between the PSA^+ and $PSA^{-/lo}$ populations is even more evident when one considers the tumours, which they recapitulate *in vivo*. Tumours

originating from a PSA-positive population contain mainly PSA^+ cells, whereas tumours derived from $PSA^{-/lo}$ cells contain a dichotomous population of cells. This is extremely characteristic of the propensity of stem cells to undergo multi-lineage differentiation and highlights the inherent biological differences, which exist between the PSA^+ and $PSA^{-/lo}$ populations. A multitude of genes associated with stem cell functions were also found to be enriched in $PSA^{-/lo}$ cells isolated from LAPC9 and LNCaP cells including Nanog, SOX15, CD44, IGF-1 and TGFBR1. Overall, when combined with previous studies identifying putative prostate cancer stem cells, these results indicate that the $PSA^{-/lo}$ phenotype may represent a heterogeneous population of tumour-initiating stem-like cells. Previous findings reporting the identification of stem-like cancer cells have demonstrated that these various populations lack PSA and the androgen receptor, which would indicate that the $PSA^{-/lo}$ population collectively represents these cellular subsets of varying tumorigenic ability (Collins *et al.,* 2005; Patrawala *et al.,* 2006).

5.1.3 Prostatospheres

A number of different systems have been employed in an attempt to culture prostate cancer stem cells *in vitro* including suspension growth, low adherence culture on agar and low adherence plates (Miki *et al.,* 2007; Gu *et al.,* 2007; Dubrovksa *et al.,* 2009). These techniques often involve the use of medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and generate one-dimensional non-adherent prostatospheres. While a number of studies have reported the enrichment of stem cell properties within these spheres, Rybak *et al.,* (2011) were the first to demonstrate long term culture of prostatospheres isolated from the highly aggressive metastatic cell line DU145.

This study demonstrated that approximately 1.25% of monolayer DU145 cells can produce spheres when grown in serum free medium supplemented with EGF. These spheres were found to exhibit a number of crucial stem characteristics including the ability to regenerate a heterogeneous cellular population as demonstrated by the expression of prostate lineage-specific markers (CD44, integrin$\alpha_2\beta_1$, and cytokeratin 18). Furthermore, these spheres were found to initiate significantly more aggressive xenograft tumours when compared to monolayer cells. Tumours derived from sphere-cells were also found to be $CD44^+$, which correlates with previous findings indicating an increased tumorigenic capacity in $CD44^+$ populations (Patrawala *et al.,* 2006). DU145 prostatospheres were found to be $CD44^+CD24^+$ which could indicate that these spheres represent a mixed population of both stem and progenitor cells. This hypothesis is further supported by findings that spheres express both basal (CD44, integrin$\alpha_2\beta_1$, 34βE12) and luminal (CK18) cell-specific markers. This

expression profile may also indicate that prostate cancer stem cells arise from a transforming event during the differentiation of normal stem to progenitor cell. It is prudent to note, that these DU145 prostatospheres were found to be CD133-negative which, contradicts previous findings identifying CD133 as a marker of stem potential. This perhaps acts as a cautionary reminder that many studies regarding the isolation and characterisation of prostate cancer stem cells yield heterogeneous results and as yet there does not exist a bona fide panel of definitive prostate cancer stem cell-markers.

A further study by Salvatori *et al.,* (2012) has demonstrated that cells of the phenotype $CD44^{+}CD24^{-}$ isolated from the DU145 cell line also possess the ability to generate non-adherent spherical colonies when cultured in serum-free medium supplemented with EGF, bFGF and insulin. These spheres were found to differentiate and grow in a monolayer upon removal of the conditioned medium and addition of 10% FBS-containing medium. Their self-renewal capacity was demonstrated upon analysis of derivative xenograft tumours as two cellular populations were identified; both differentiated cells which formed the bulk of the tumour and $CD44^{+}CD24^{-}$ cells. Furthermore, putative CSC-containing spheroids were found to be decidedly more tumorigenic than parental DU145 cells, yielding highly aggressive, metastatic tumours. Again, these findings are consistent with data published by Patrawala *et al.,* (2006), which reported the highly tumorigenic capacity of CD44-positive prostate cancer cells. Interestingly, Salvatori *et al.,* (2012) noted that the ability of spheroids to generate highly-vascularised, aggressive tumours was somewhat dampened upon injection of spheres in combination with differentiated parental DU145 cells. These findings would appear to suggest that the cancer stem cell population is sensitive to environmental signals from differentiated cells, which may ultimately determine tumour aggressiveness. While this anomaly has only been demonstrated in one prostate cancer stem cell model, it provides an attractive mechanism for the widespread treatment failure, which poses such a clinical burden in prostate cancer. Perhaps, the removal of the bulk population of differentiated cells by conventional treatment strategies is infact facilitating tumour regeneration. However, additional experimentation is required in order to further explore this hypothesis.

5.1.4 Embryonic Pluripotency Markers

Embryonic stem cell pluripotency factors such as NANOG, OCT4 and SOX2 are known to regulate a number of innate stem cell features including self-renewal. As many of these features are also ascribed to cancer stem cells, it has been postulated that the expression patterns of these genes may be useful in the identification of tumorigenic stem-like cells. Indeed, a study by Ben-Porath *et al.,* (2008) has demonstrated that poorly differentiated tumours preferentially overexpress a number of genes associated with embryonic stem cell

identity. Furthermore, these genes including NANOG, OCT4, SOX2 and c-MYC are more frequently observed in poorly-differentiated tumours than in well-differentiated tumours indicating that the histopathological traits of a tumour are determined by the concerted activity of these genes, however it remains unsettled the extent to which these factors contribute to tumour aggressiveness.

With regards to prostate cancer, prostatospheres derived from the DU145 cell line were found to express NANOG and OCT4 at a level similar to monolayer parental cells, however the expression of SOX2 was found to be enriched in spheres alone (Rybak *et al.,* 2011). The expression of NANOG and OCT4 was also found to directly correlate with increasing Gleason grade in primary human prostate tumours (Mathieu *et al.,* 2011). Furthermore, a study by Germann *et al.,* (2012) has demonstrated that in novel androgen-dependent human prostate cancer BM18 xenograft tumours, stem-like cells highly express NANOG as well as the putative stem marker ALDH1A1 (aldehyde dehydrogenase 1A1) and luminal markers CK18 and NKX3.1. These results underscore the potential role of castration-resistant stem-like cells in treatment failure.

Taken together, these findings highlight the relevance of pluripotentiality to the identification of prostate cancer stem cells and the utility of pluripotency factors in the confirmation of stem identity.

5.1.5 Prostate Cancer Holoclones

As mentioned in 5.1, seminal work by Barrandon and Green established the relationship between stem potential and colony forming ability (Barrandon and Green, 1987). The capacity of this colony forming assay to demonstrate the phenotypic and proliferative diversity present within somatic tissue has since been exploited to identify and characterise normal stem cells in many cultured cell lines, including skin, follicular and limbal tissue (Mackenzie, 2005). Due to the apparent parallels which exist between normal and cancer stem cells, the generation of morphologically heterogeneous colonies with varying proliferative abilities known as holoclones, meroclones and paraclones has been adopted as a surrogate assay for the identification of cancer stem cells and there is increasing evidence to support the presence of cancer stem cells within cancer cell holoclones. This technique has been employed to identify stem-like cells expressing critical self-renewal genes in multiple human cancer types, including pancreatic (Tan *et al.,* 2011), head and neck (Harper *et al.,* 2007), breast (Liu *et al.,* 2013) and prostate (Locke *et al.,* 2005; Li *et al.,* 2008). Monoclonal cultivation/colony forming assay involves the culture of cells at single-cell densities and is predicated on the assumption that only rare cells, which possess the capacity to self-renew, will yield large colonies.

Li *et al.*, (2008) have demonstrated that the intrinsic heterogeneity present within somatic tissue is also observed in immortalised human prostate carcinoma PC-3 cells. This study employed single-cell propagation by limiting dilution to demonstrate that human prostate cancer PC-3 cells behave much like primary human keratinocytes under such conditions. Approximately two weeks following plating at single-cell titres, PC-3 cells gave rise to morphologically heterogeneous colonies resembling the holoclones, meroclones and paraclones described in Barrandon & Green's original work (1987). These distinct colonies were found to possess significant developmental and functional plasticity, which closely correlated with the hierarchical proliferative stem and amplifying patterns observed in the normal epithelial compartment. In addition, PC-3 holoclones were found to preferentially express a number of key stem cell markers including CD44, integrin$\alpha_2\beta_1$, and β-catenin, while paraclones exhibited undetectable expression of all three markers. These findings indicate that colony forming assay may represent a suitable method of *in vitro* cancer stem cell identification.

To date, results derived from investigations into cancer stem cell identity and function have provided an abundance of evidence to substantiate the existence of a rare subpopulation of self-renewing, tumour-initiating, stem-like cells. A deeper understanding of the mechanisms which dictate cancer stem cell behaviour will surely facilitate the development of novel therapeutics, which effectively target this population, thus potentially eliminating the widely recognised burden of therapy failure in prostate cancer.

5.1.6 Experimental Hypothesis and Aims

It has been postulated that a subpopulation of cancer stem cells drives tumour growth and that this cellular populace is responsible for treatment failure and the subsequent development of hormone-refractory prostate cancer. The hypothesis of this work was that intrinsic stem cell characteristics such as self-renewal are retained in long-term cultured epithelial cell lines and that these putative stem-like cells can be isolated to provide an efficacious *in vitro* model for the study of malignant stem cells.

The primary aims of these investigations were to address the relative dearth of understanding regarding the biologic properties of prostate cancer stem cells by optimising a number of methods of prostate cancer stem cell isolation and subsequently characterising the differential gene expression repertoires of these putative stem-like cells by analysing a panel of established prostate cancer-specific and canonical stem markers.

The methods of prostate cancer stem cell isolation we sought to investigate included;

- FACS-identification of a population displaying the $CD44^{+}$/integrin$\alpha_2\beta_1^{hi}$/$CD133^{+}$ antigenic phenotype.

- Low-adherence culture on high-salt soft agar, a technique previously optimised within our laboratory.

- Prostatosphere derivation through culture in serum-free medium supplemented with insulin, human recombinant fibroblast growth factor (FGF) and human recombinant epidermal growth factor (EGF).

- Morphologically heterogeneous colony forming assay.

We sought to construct a specific panel of stem markers for investigation, which included a number of previously identified prostate cancer-specific markers including CD44, CD24, integrin$\alpha_2\beta_1$, ALDH1 (ALDH1A1), integrinα6, and the proto-oncogene c-met. Due to the previously documented utility of embryonic pluripotency stem markers in the identification of cancer stem cells, we also sought to examine the expression of NANOG, POU5F1/OCT4 and SOX2.

5.2 Results

5.2.1 Determination of Putative Stem Cell Fraction in Cell Line Panel

The first aim of this study was to employ fluorescent activated cell sorting (FACS) to determine the proportion of cells within each cell line, which represented the putative stem cell fraction. In order to achieve this, the expression of an antigenic signature previously reported to be associated with stem properties; $CD44^{+}$/integrin$\alpha_2\beta_1^{hi}$/$CD133^{+}$ was examined. This signature was found to be differentially expressed across our cell line panel; however its levels of expression closely correlated with previous findings, indicating that only a small fraction of the cellular population are enriched for cancer stem cell properties.

Of the four cell lines assayed, only a small subset of each was found to coexpress all three surface markers. 2.05 % of the DU145 population were found to be triple positive for these markers (Figure 5.1), while integrin$\alpha_2\beta_1$ and CD44 were highly expressed by all populations within this cell line (Table 5.1), thus CD133 expression was restricted to a small fraction of cells. This trend followed for the PC-3 and 22Rv1 cell lines with 2.3 % and 3.47 % respectively of the cells expressing the full $CD44^{+}$/integrin$\alpha_2\beta_1^{hi}$/$CD133^{+}$ signature (Figure 5.2, Figure 5.3). LNCaP cells were found to express a higher level of these surface antigens with approximately 16.9 % of the cellular population coexpressing all three surface markers (Figure 5.4).

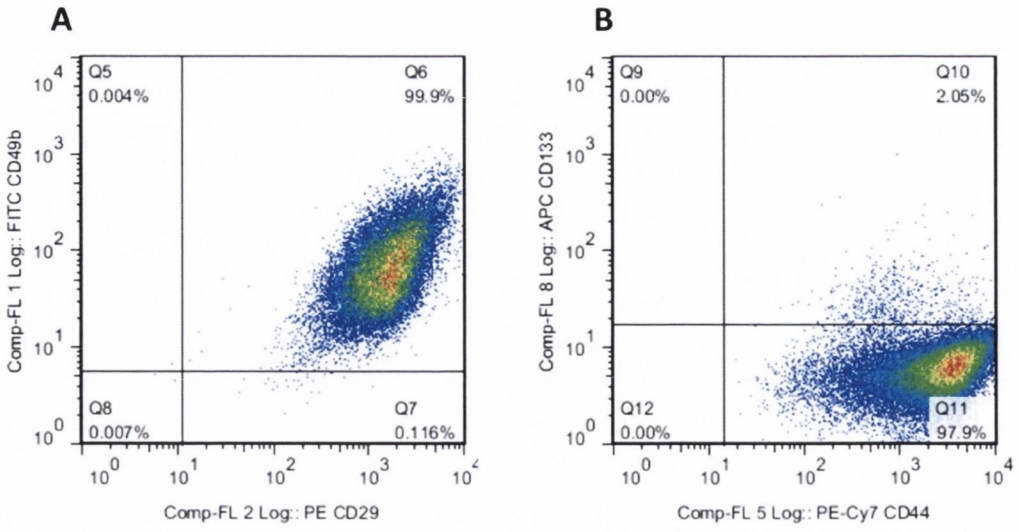

Figure 5.1 *Flow cytometric dot plots of integrin$\alpha_2\beta_1$, CD44 and CD133 expression in DU145 parental cell line.*

(A) 100% of the DU145 population were found to be positive for integrin$\alpha_2\beta_1$ expression. (B) CD44 was also very highly expressed, however only a small population of cells (2.05%) were found to coexpress all three surface markers.

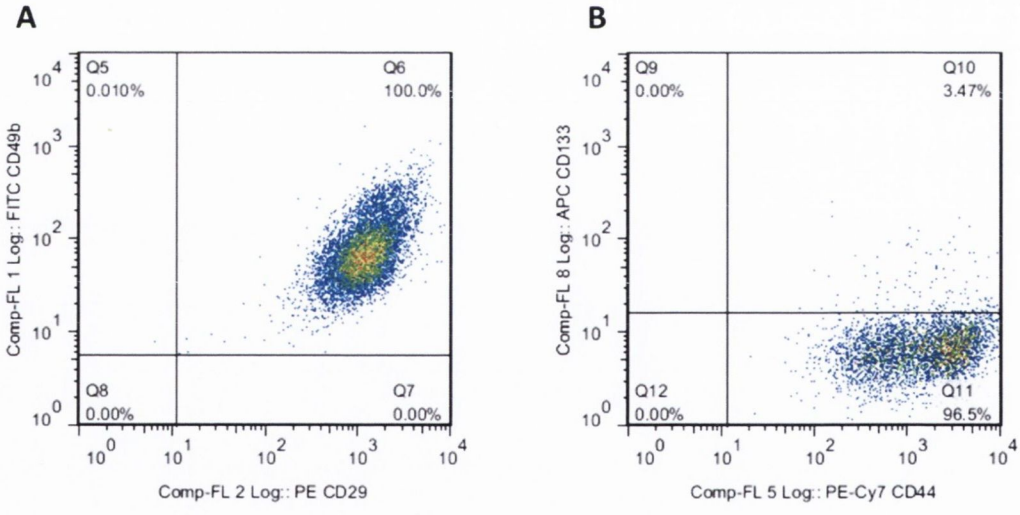

Figure 5.2 *Flow cytometric dot plots of integrin$\alpha_2\beta_1$, CD44 and CD133 expression in PC-3 parental cell line.*

(A) 100% of the cells express the surface marker integrin$\alpha_2\beta_1$. (B) A large proportion of cells express CD44, while only 3.47% of cells appear to coexpress CD133 and CD44.

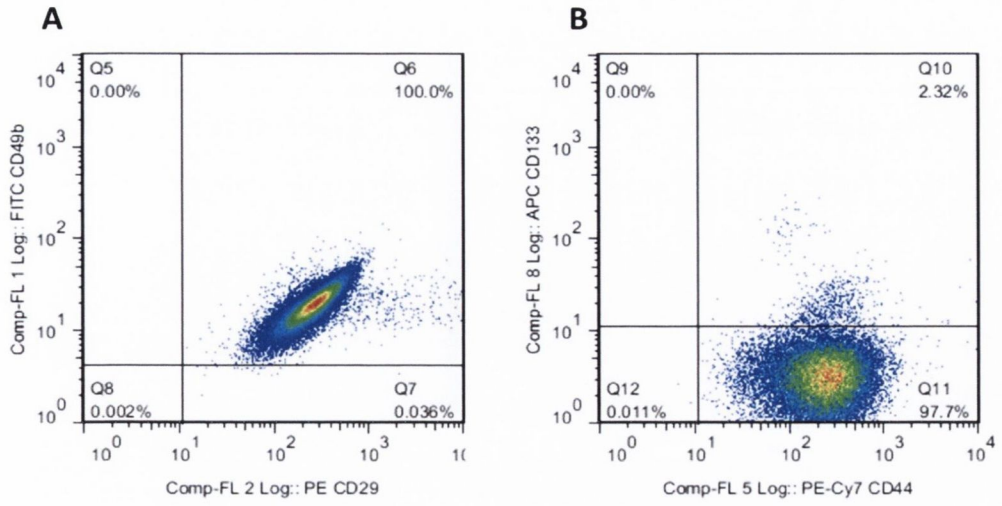

Figure 5.3 *Flow cytometric dot plots of integrin$\alpha_2\beta_1$, CD44 and CD133 expression in 22Rv1 parental cell line.*

(A) Similar to the expression patterns observed in the PC-3 cell line, 100% of the cell population express integrin$\alpha_2\beta_1$. (B) CD44 is expressed by 97.7% of 22Rv1 cells, however only a small percentage of cells (2.32%) simultaneously express CD133.

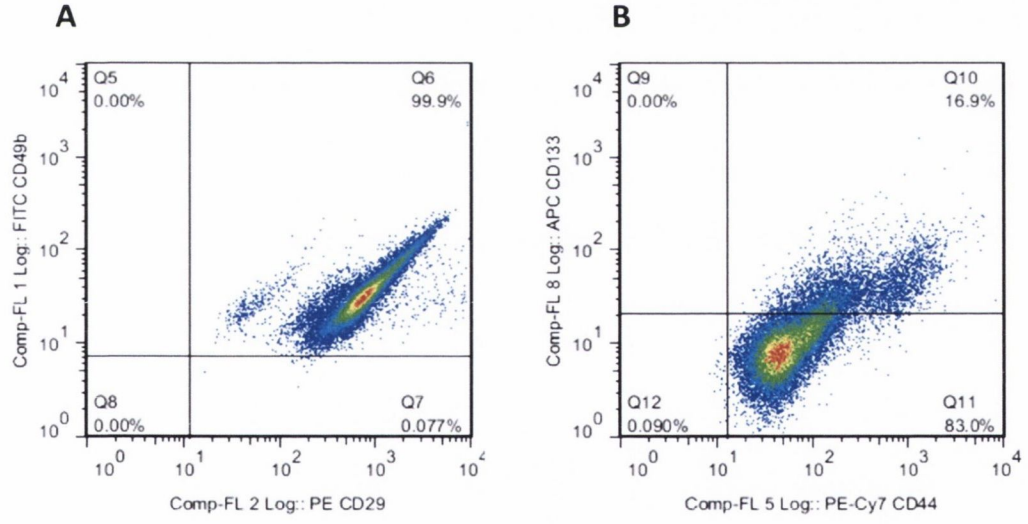

Figure 5.4 *Flow cytometric dot plots of integrin$\alpha_2\beta_1$, CD44 and CD133 expression in LNCaP parental cell line.*

(A) LNCaP cells possess the same integrin$\alpha_2\beta_1$ expression pattern as both PC-3 and 22Rv1, with close to 100% of the population being positive for the surface marker. (B) However a slightly larger population of cells (16.9%) are positive for CD133 expression than in 22Rv1 and PC-3 cells.

Marker	22Rv1 % expression	LNCaP % expression	PC-3 % expression	DU145 % expression
CD49b (integrinα_2)	100	100	100	100
CD29 (integrinβ_1)	100	100	100	100
CD44	97.7	83	96.5	97.9
CD133	2.3	16.9	3.47	2.05

Table 5.1 Percentage expression of $CD44^+$/integrin$\alpha_2\beta_1^{hi}$/$CD133^+$ signature across cell line panel.

5.2.2 High Salt Agar Assay

In order to enrich for cells of a putative stem phenotype, a technique previously optimised within our laboratory was initially employed. This method exclusively selects for the growth of cancer cell holoclones. Previous work performed both in our laboratory (using melanoma and thyroid cell lines) and elsewhere has demonstrated that low-density culture of cells on a high sodium surface selectively generates colonies of a holoclone morphology, which are postulated to contain stem-like cells (Olszewski *et al.,* 2005). This technique was applied to our prostate carcinoma cell line panel. PC-3, 22Rv1, LNCaP and DU145 prostate cancer cells were found to exclusively generate putative stem cell-containing holoclones when grown on high NaCL-concentration agarose medium. Cells were resuspended in complete media and plated at low-density (1 x 10^6 cells) on a 1% agarose NaCL surface and holoclones were observed at differential time points following initial plating for each cell line (Figure 5.5). Holoclones were observed as densely arranged bundles of cells, spherical in morphology due to the presence of a defined halo structure surrounding the cells in the agarose surface (Figure 5.6, Figure 5.7, Figure 5.8, Figure 5.9). Cells developed within this halo and generally grew to its boundaries, however they were not observed growing beyond the perimeter of this structural anomaly. It is prudent to note, that while 22Rv1 and LNCaP parental cells generated holoclones more efficiently than PC-3 and DU145 cells, they rarely grew to capacity. The precise function of this apparent microvesicle has yet to be elucidated; however it does not appear to be unique to prostate cancer cells as it has previously been observed within our laboratory for melanoma, thyroid and ovarian cancer cell lines.

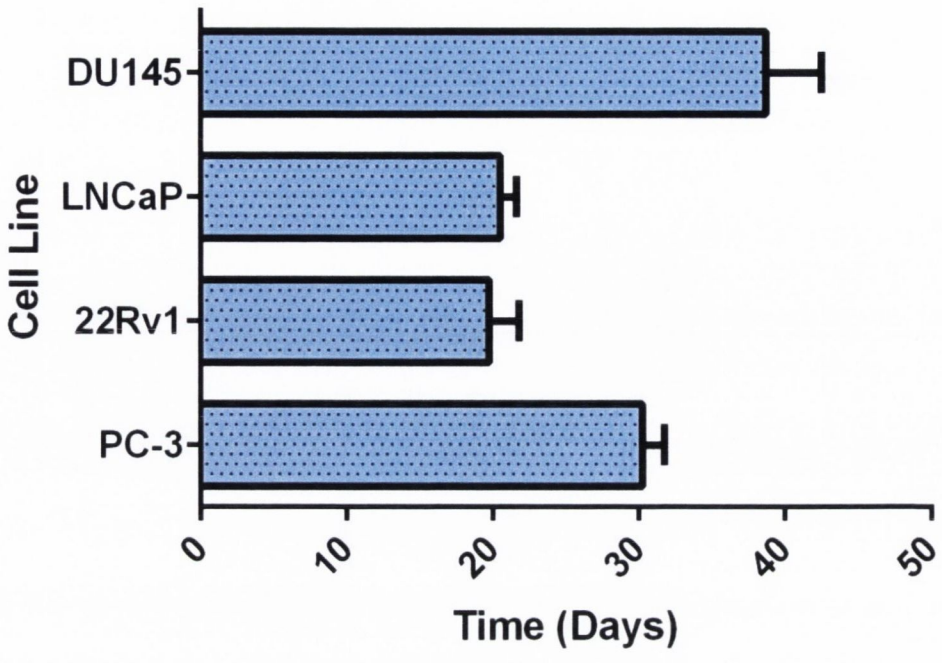

Figure 5.5 *High salt agar holoclone generation efficiencies (n=3).*

Both 22Rv1 and LNCaP parental cells yielded holoclones ~3-4 weeks following plating on agarose medium, while holoclone morphology was not identified in PC-3 and DU145 cells until 4 and 6 weeks respectively. Time is measured upon first holoclone appearance per cell line.

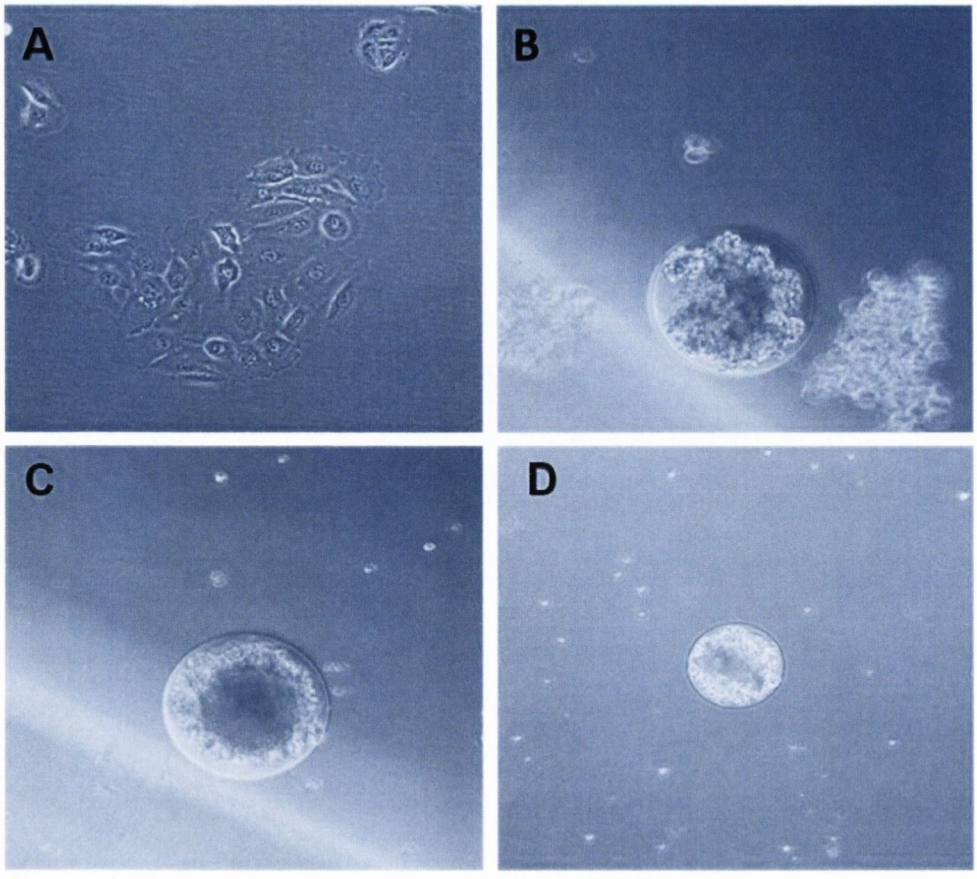

Figure 5.6 *DU145 differentiated parental cells and derivative holoclones.*

(A) DU145 parental cells. (B) DU145 early holoclone (~ 6 weeks following plating). (C) DU145 intermediate holoclone. (C) DU145 full holoclone at ~ 7 weeks following initial plating (20X objective).

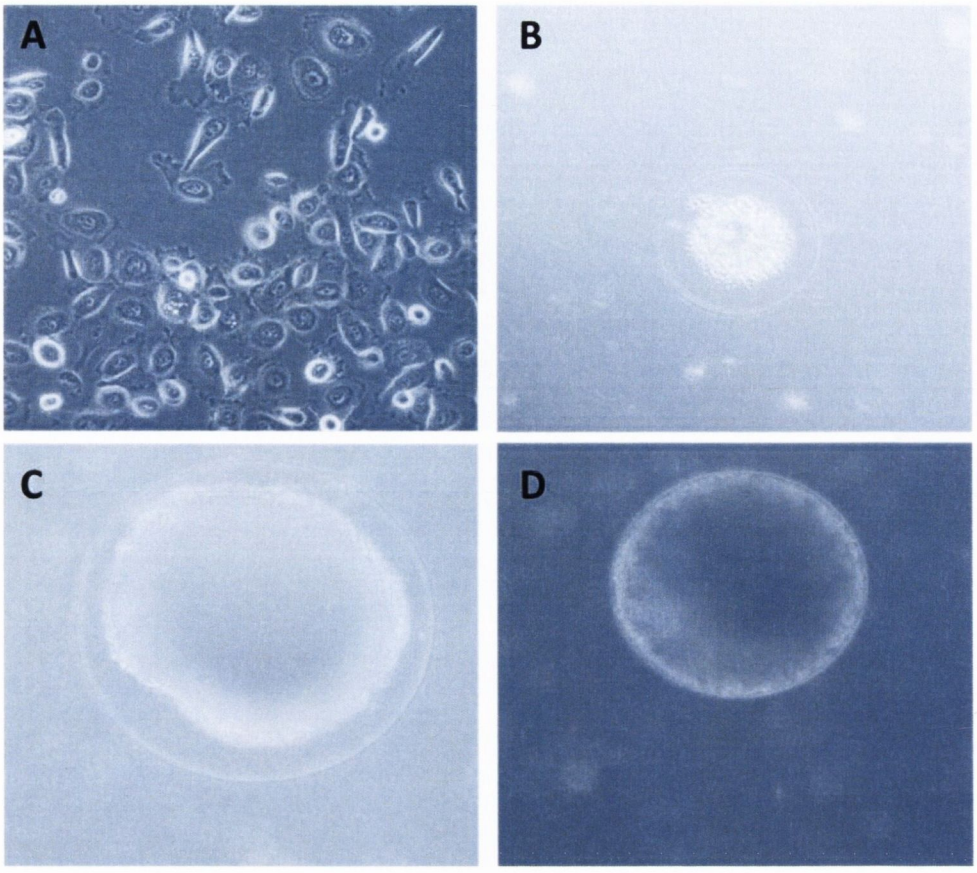

Figure 5.7 *PC-3 parental cells and derivative holoclones.*

(A) PC-3 parental cells. (B) PC-3 early holoclone observed ~ 4 weeks post-plating. (C) PC-3 holoclone of intermediate size. (D) PC-3 holoclone at maximum growth ~ 6 weeks following plating (20X objective).

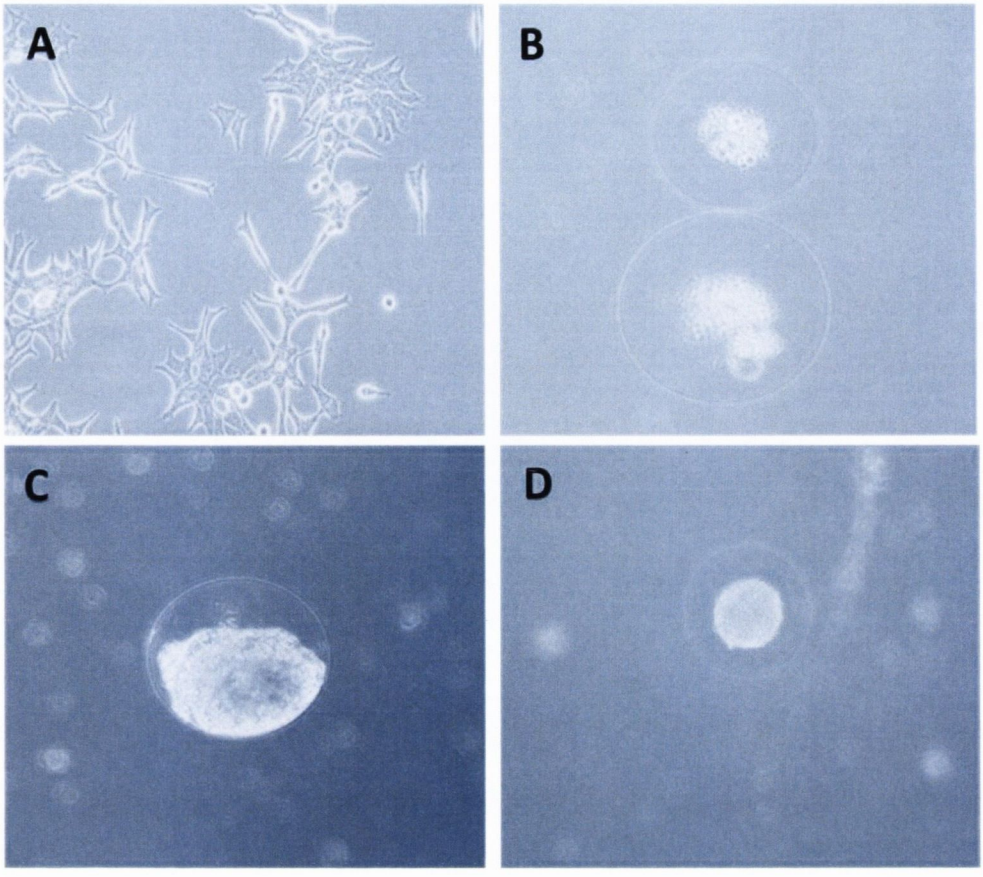

Figure 5.8 *LNCaP parental cells and derivative holoclones.*

(A) LNCaP parental cells. (B) Two LNCaP holoclones growing side by side upon first inspection ~ 3 weeks following plating. (C) LNCaP holoclone at full size ~ 5 weeks following plating. (D) LNCaP holoclone at full size ~ 5 weeks following plating (20X objective).

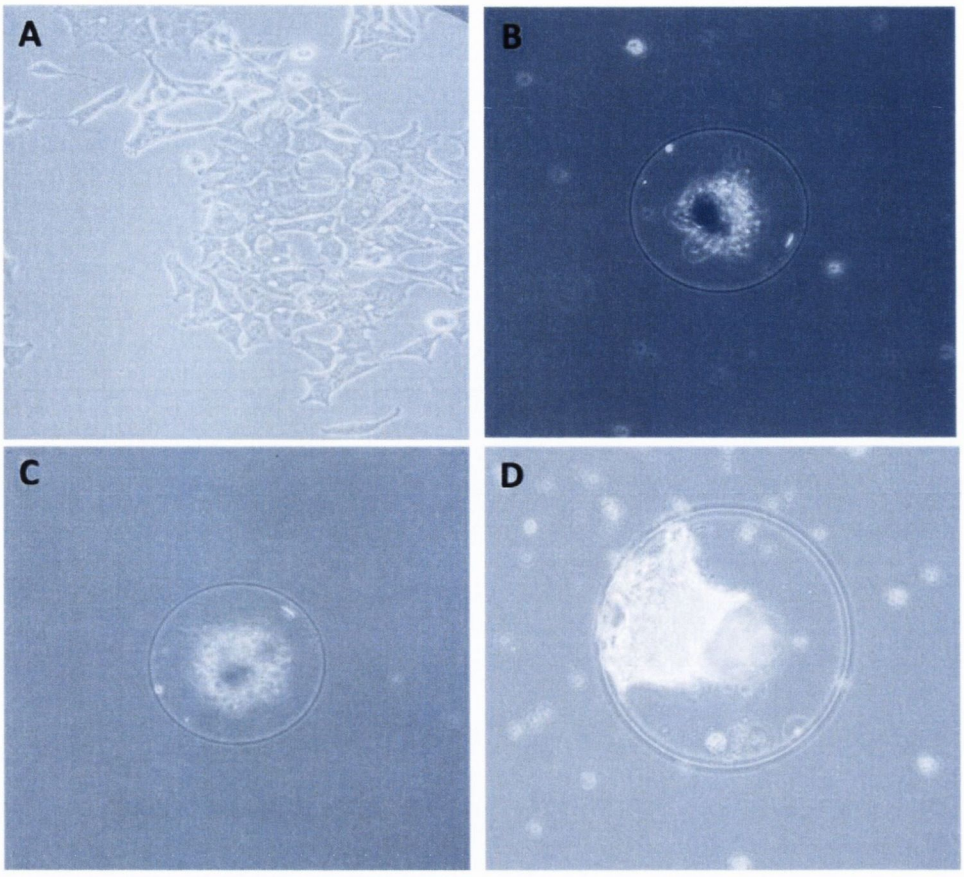

Figure 5.9 *22Rv1 parental cells and derivative holoclones.*

(A) 22Rv1 parental cells. (B) 22Rv1 early holoclone ~ 3 weeks following plating. (C) 22Rv1 intermediate holoclone. (D) 22Rv1 holoclone at maximum growth ~ 5 weeks following plating (20X objective).

5.2.3 Interrogation of Stem Potential in High Salt Agar-derived Holoclones

The putative stem phenotype of holoclones generated using the high salt agar technique was assessed by examining the expression of a panel of key stem cell-associated genes using quantitative RT-PCR. Samples were normalised to the relevant parental cell line. This analysis yielded highly variable expression patterns amongst individual holoclone replicates originating from the same cell line and also between cell lines. For example, aldehyde dehydrogenase 1A1 (ALDH1/ALDH1A1), whose increased expression has been identified in the stem cell populations of various solid tumour types, exhibited low or undetectable expression levels (C_t > 35) in both DU145 and PC-3 holoclones (Ginestier *et al.,* 2007). Furthermore, ALDH1 was found to be statistically significantly downregulated in LNCaP holoclones when compared to the resting parental counterpart (Student's two-tailed t-test, p-value <0.05). Conversely, ALDH1 expression was found to be consistently overexpressed in 22Rv1 holoclones. The surface antigen CD133 followed a similar variable expression pattern. It was found to be overexpressed in DU145 holoclones, however it was downregulated in LNCaP holoclones and exhibited undetectable expression levels in PC-3 and 22Rv1 holoclones. Both CD24 and CD44 were found to be consistently overexpressed in holoclones across the cell line panel. The integrin$\alpha_2\beta_1$ subunits were found to be overexpressed in PC-3 and DU145 holoclones; however their expression levels in LNCaP and 22Rv1 holoclones were comparable to that of the parental cell lines. Integrinα_6 expression also followed this pattern, it was found to be overexpressed in PC-3 and DU145-derived holoclones; however its expression did not differ greatly from resting parental cells in both 22Rv1 and LNCaP holoclones. The proto-oncogene c-met was overexpressed in PC-3 and DU145 holoclones; however it was statistically significantly downregulated in LNCaP-derived holoclones ($p = 0.02$). No difference in its expression was observed between 22Rv1 parental cells and their resultant holoclones. NANOG was found to be statistically significantly overexpressed in PC-3 ($p = 0.03$) and 22Rv1 holoclones ($p = 0.04$). The expression of POU5F1/OCT4 was statistically significantly overexpressed in PC-3 holoclones ($p = 0.02$); however it was found to display consistently higher expression than parental cells in LNCaP and 22Rv1 holoclones. Gene expression patterns of high salt agar-derived holoclones are summarised in Table 5.2.

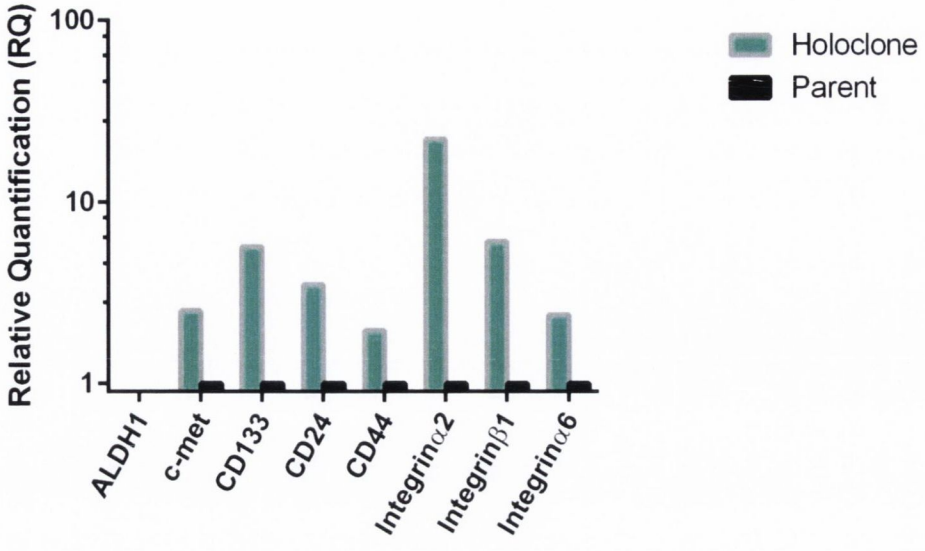

Figure 5.10 *Stemness gene expression analysis in DU145 holoclone (n=1).*

Stem cell-associated genes were found to be upregulated in DU145 holoclones when compared to their differentiated parental counterparts. Note: Due to the poor efficiency of the DU145 cell line to generate holoclones on high salt agar only one replicate was available for analysis of eight target genes.

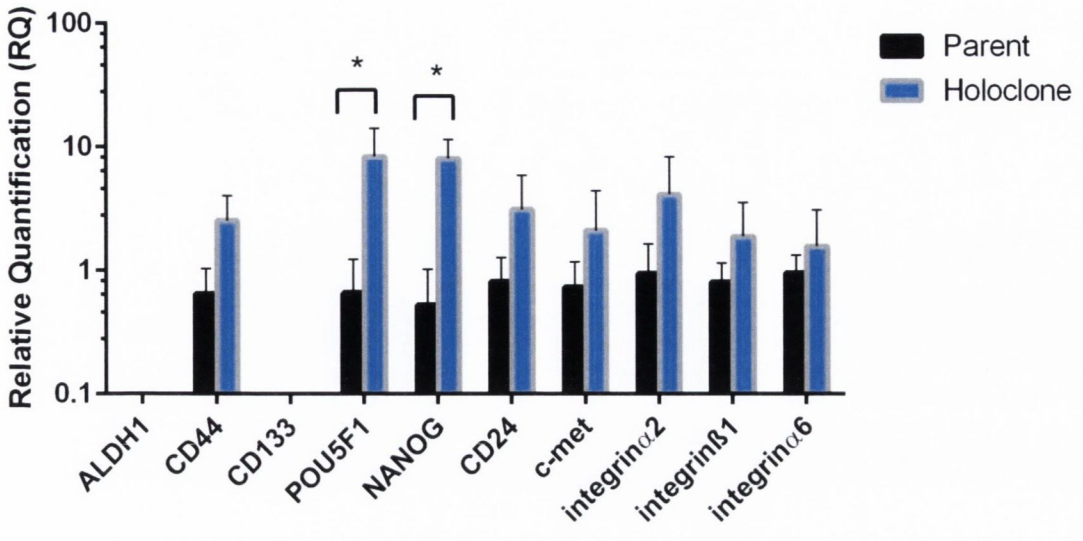

Figure 5.11 *Stemness gene expression analysis in high salt agar-derived PC-3 holoclones (n=3).*

Relative quantification (RQ) of change in expression of stem-associated genes in holoclones normalised to parental control. NANOG and OCT4/POU5F1 are statistically significantly overexpressed in holoclones when compared to differentiated parental cells. No bars indicate low or undetectable expression of target in holoclone samples. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

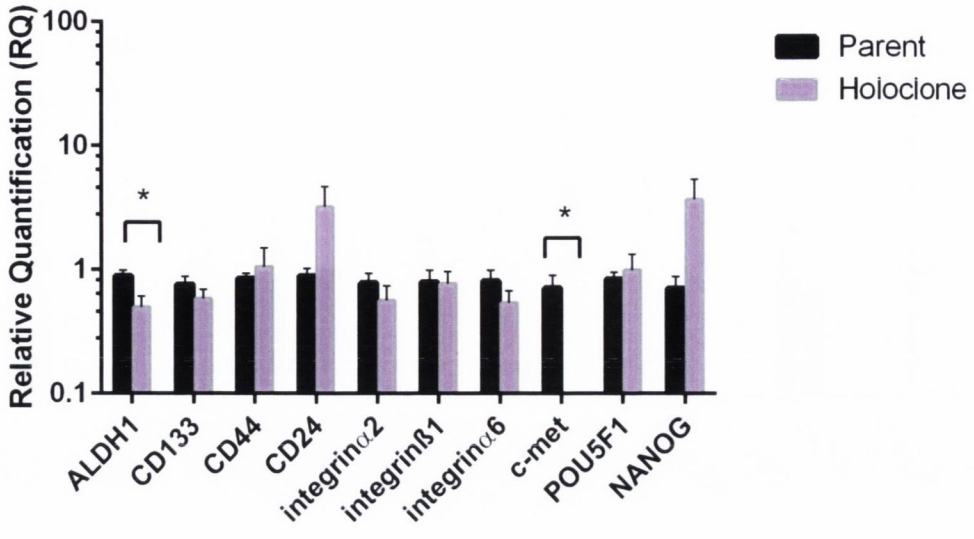

Figure 5.12 *Stemness gene expression analysis in high salt agar-derived LNCaP holoclones (n=3).*

Relative quantification (RQ) of change in expression of stem-associated genes in holoclones normalised to parental control. ALDH1 and c-met are statistically significantly downregulated in LNCaP holoclones when compared to differentiated parental cells. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

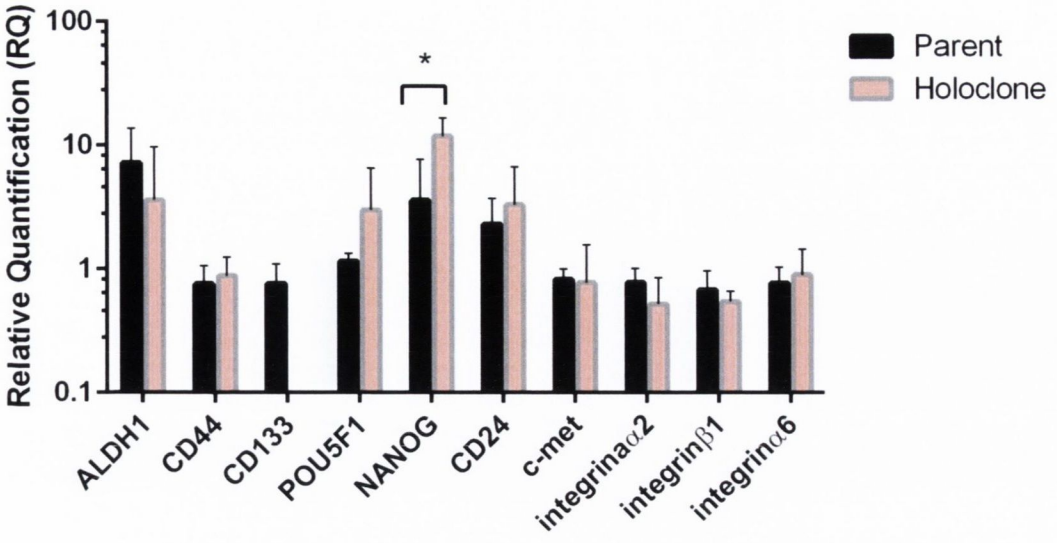

Figure 5.13 *Stemness gene expression analysis in high salt agar-derived 22Rv1 holoclones (n=3).*

Relative quantification (RQ) of change in expression of stem-associated genes in holoclones normalised to parental control. NANOG is statistically significantly overexpressed in 22Rv1 holoclones when compared to differentiated parental cells. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

Gene Target	Cell Line and Expression in Derivative Holoclones			
	DU145	**PC-3**	**22Rv1**	**LNCaP**
ALDH1	**Undetectable**	**Undetectable**	↓	↓*
CD44	↑	↑	↑	↑
CD24	↑	↑	↑	↑
CD133	↑	**Undetectable**	↓	↓
Integrinα2	↑	↑	↓	↓
Integrinβ1	↑	↑	↓	↓
Integrinα6	↑	↑	↑	↓
c-met	↑	↑	↓	↓*
OCT4/POU5F1	-	↑*	↑	↑
NANOG	-	↑*	↑*	↑

Table 5.2 *Table of stem-associated gene expression patterns in high salt agar-derived holoclones.*

↑ denotes upregulation of target gene in holoclone normalised to relevant parental cell line. ↓ denotes downregulation of target gene in holoclone normalised to relevant parental cell line. Note: OCT4/POU5F1 and NANOG were not assayed in DU145-derived holoclones. *denotes statistical significance.

5.2.4 Generation of Prostatospheres

The ability of PC-3 and DU145 cells to generate three-dimensional prostatospheres was assayed by culture in serum-free medium supplemented with EGF, bFGF, BSA and insulin (Wintzell *et al.*, 2012). The cells were seeded at low density (1 x 10^4) in T25 flasks and their growth observed over 14 days. Neither PC-3, nor DU145 cells generated morphologically unique spheres. Both cell lines displayed growth patterns equivalent to culture in standard medium. However, cells were harvested at 14 days and their stem phenotype assessed by examining the expression of the stem-associated gene panel. Samples were normalised to the relevant parental cell line. OCT4/POU5F1 (p= 0.01), NANOG (p= 0.002), and SOX2 (p= 0.001) were found to be statistically significantly upregulated in DU145 cells grown in stem cell medium (Figure 5.14). CD44 and CD133 were also upregulated but did not reach significance. ALDH1 was found to be downregulated in DU145 'sphere' cells. All of the stem-associated genes were upregulated in PC-3 cells grown in stem cell medium; however they did not reach significance (Figure 5.15).

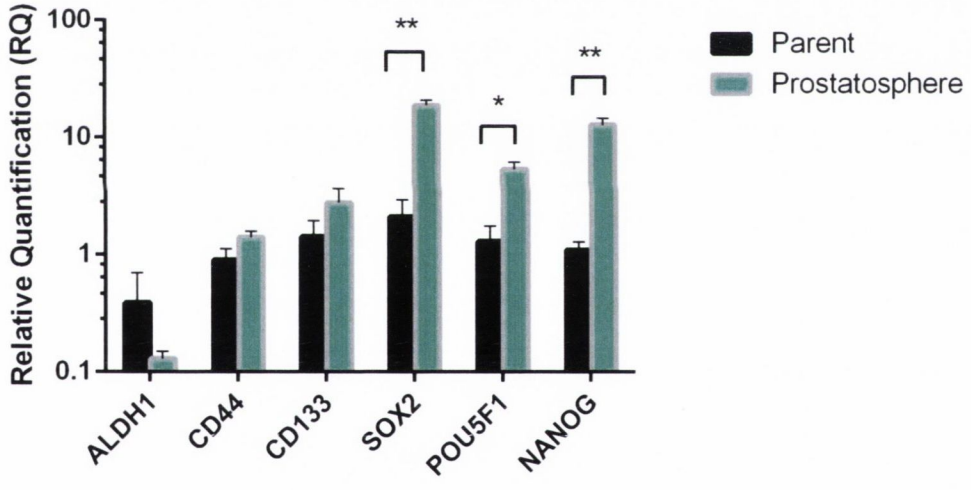

Figure 5.14 *Stemness gene expression analysis in DU145 'prostatospheres' (cells cultured in stem cell medium) (n=3).*

Relative quantification (RQ) of change in expression of stem-associated genes in holoclones normalised to parental control. SOX2, OCT4/POU5F1 and NANOG are statistically significantly overexpressed in DU145 prostatospheres when compared to differentiated parental cells. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

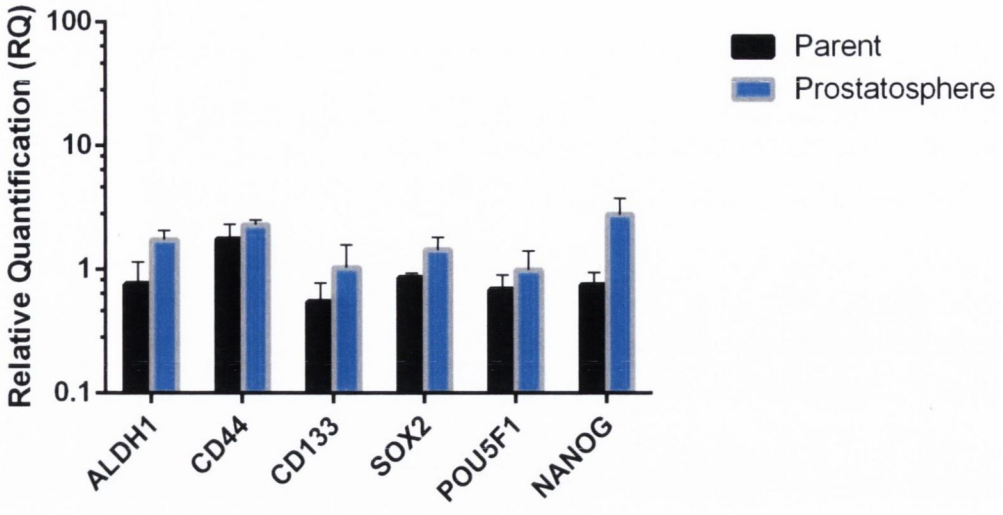

Figure 5.15 *Stemness gene expression analysis in PC-3 'prostatospheres' (cells cultured in stem cell medium) (n=3).*

Relative quantification (RQ) of change in expression of stem-associated genes in holoclones normalised to parental control. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

5.2.5 PC-3 and DU145 Cells Generate Morphologically Heterogeneous Colonies

The adoption of monoclonal cultivation or colony forming assay as a surrogate technique for the isolation of putative cancer stem cells has proved successful in a number of immortalised cancer cell lines. The aim of this portion of the study was to apply the monoclonal cultivation technique to our cell line panel in order to assess its ability to; a) efficiently enrich for putative cancer stem cell-containing holoclones and b) generate a cancer stem cell fraction on a larger-scale for downstream analyses. PC-3, DU145, 22Rv1 and LNCaP cells were plated at clonal densities on 96-well plates pre-loaded with the appropriate warmed complete medium. In order to ensure that only one cell was seeded into each well, the cells were flow sorted using a MoFlo Single Cell Sorter and stringent gates were established to avoid doublets or clusters of cells. Approximately 5-7 days following seeding, adherent colonies with distinct morphologies were observed, notwithstanding a portion of cells died. Despite numerous rounds of plating 22Rv1 and LNCaP parental cells failed to yield colonies and so they were removed from our cell line panel for this aspect of the project.

When plated at single-cell titres, PC-3 and DU145 cells were found to exhibit a diversity of clonal morphologies, which closely parallel those produced by stem and late-amplifying cells of the normal epithelia. These phenotypically heterogeneous colonies could be classified as holoclones, meroclones and paraclones based on their diverse morphological features (5.1). Holoclones, which are postulated to contain self-renewing cancer stem cells, were comprised of small, tightly packed cells and possessed smooth, defined colony borders. Paraclones (comprising terminally differentiated cells) contained dispersed larger cells, while meroclones possessed an intermediate morphology containing a dichotomy of cell shapes and sizes. Their borders were often more fragmented than those observed in holoclones. The distinct morphologies observed shortly after seeding were maintained as the colonies grew. PC-3 holoclones initially grew as a monolayer, however approximately 7 days following plating it was noted that their growth graduated to a bilayer. Conversely, DU145 holoclones maintained a monolayer composition until time of harvest (t=14 days).

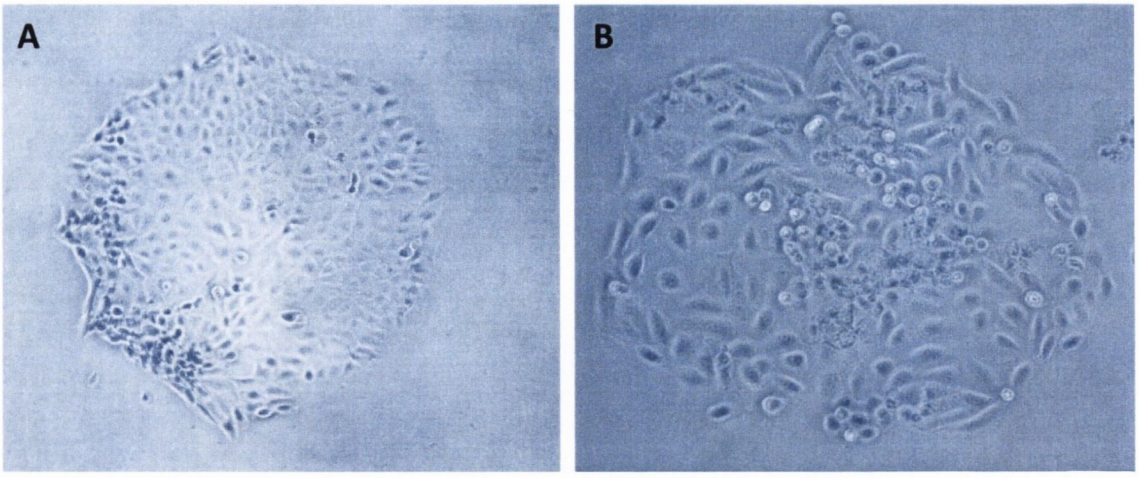

Figure 5.16 *DU145 and PC-3 early holoclones.*

(A) DU145 holoclone 5 days following plating (20X objective). (B) PC-3 holoclone 5 days following plating (20X objective).

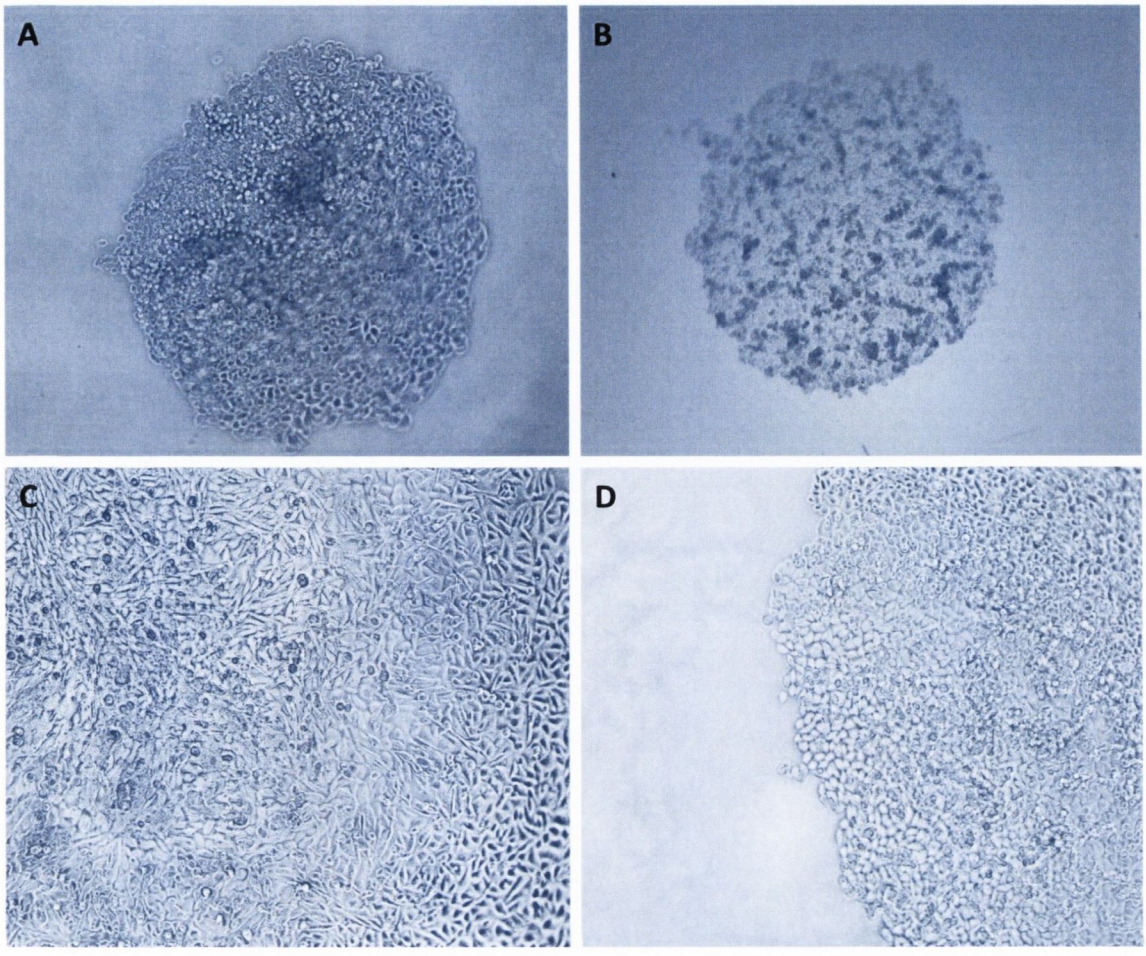

Figure 5.17 *PC-3 holoclones.*

(A) PC-3 holoclone 7 days following plating (10X objective). (B) PC-3 holoclone 10 days following plating, note the smooth colony border and dense cellular composition. The darker cells indicate that the holoclone has ceased monolayer growth and begun to grow three-dimensionally in a bilayer (10X). (C) Inner cellular composition of PC-3 holoclone (20X). (D) Borderline composition of PC-3 holoclone (20X).

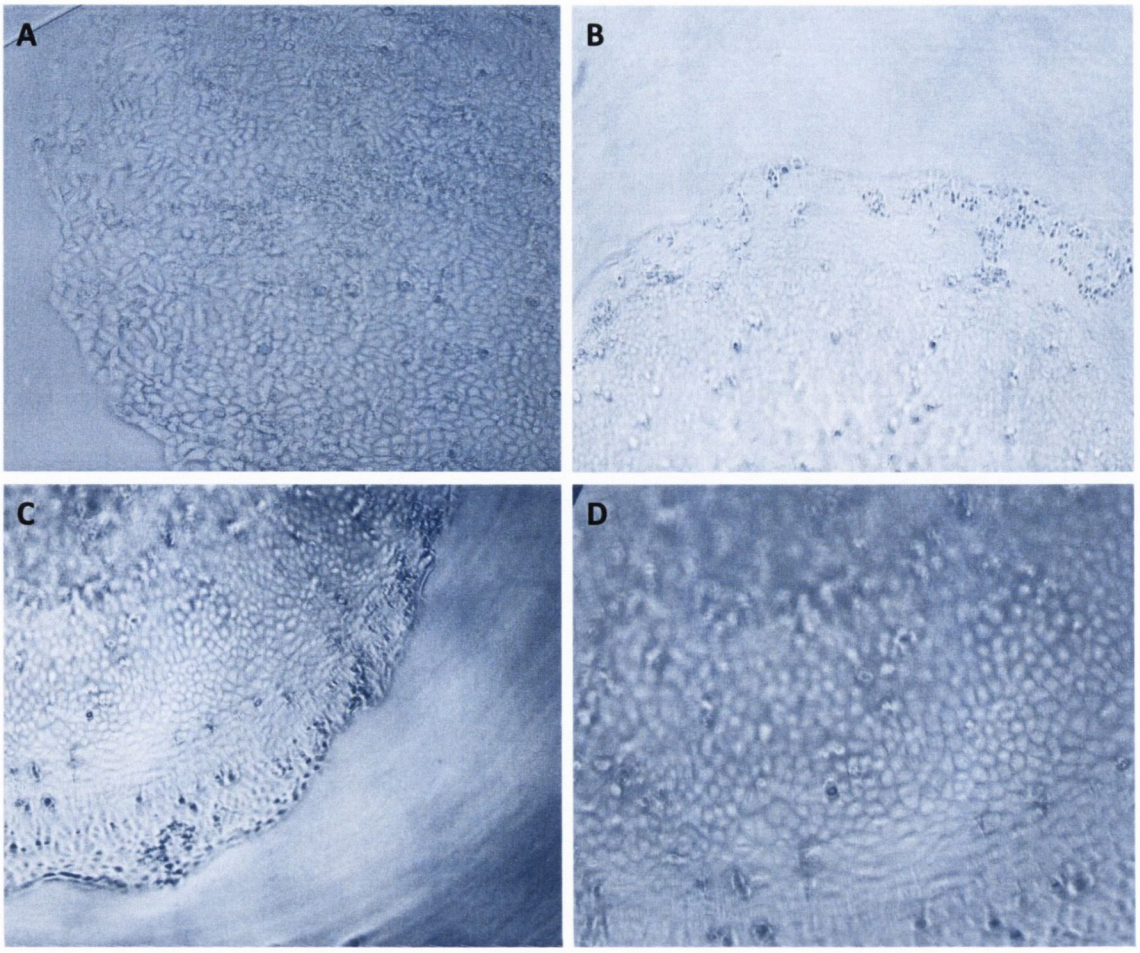

Figure 5.18 *DU145 holoclones.*

A) DU145 holoclone 7 days following plating. Note the highly arranged cellular composition and uniform colony border (10X objective). (B) DU145 holoclone perimeter (10X). (C) DU145 holoclone perimeter (10X) (D) Inner cellular composition of DU145 holoclone (20X objective).

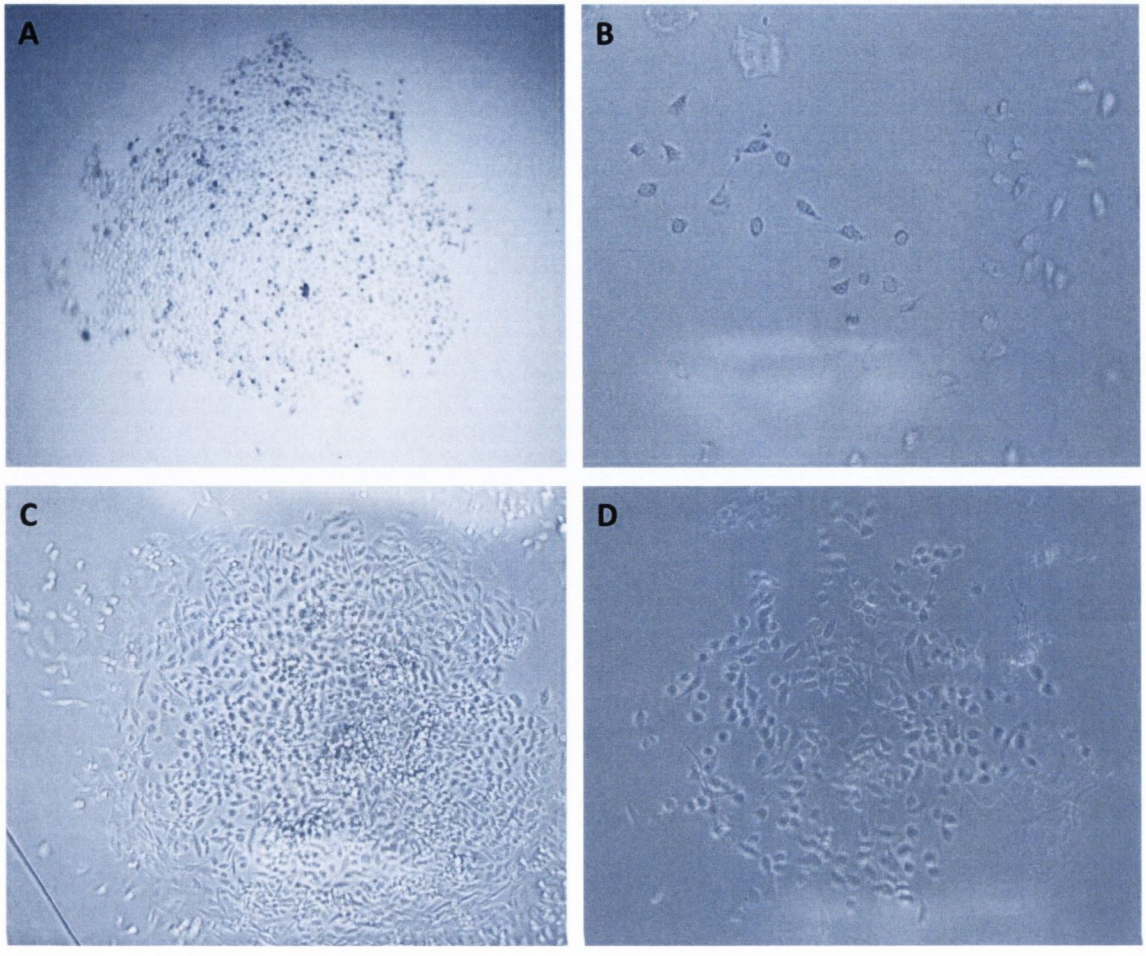

Figure 5.19 *PC-3 and DU145 meroclones and paraclones.*

(A) PC-3 meroclone exhibiting characteristic fragmented borders and loose cellular composition (10X objective). (B) PC-3 paraclone (10X). (C) DU145 meroclone (10X). (D) DU145 paraclone (10X).

5.2.6 PC-3 and DU145 Cells Exhibit Differential Propensities to Efficiently Generate Heterogeneous Colonies

PC-3 and DU145 cells were found to exhibit disparate abilities to generate phenotypically plastic colonies. Across three biological replicates (n=15 plates/replicate), 20% of the colonies produced by PC-3 cells comprised of holoclones (Figure 5.20), while the majority of colonies produced (64%) were meroclones. Paraclones were found to generate at a rate similar to holoclones (16%). Conversely, under the same parameters, 43% of the total colonies produced by DU145 cells constituted holoclones (Figure 5.21). Furthermore, meroclones were produced at a rate similar to holoclones (44%) while paraclones comprised 13 % of the colony total. Comparison of these figures to overall ability of total cells plated to yield colonies (Figure 5.22), demonstrates that approximately 1.85% of the DU145 cellular population generates holoclones, while 3.1% of the PC-3 population generates holoclones.

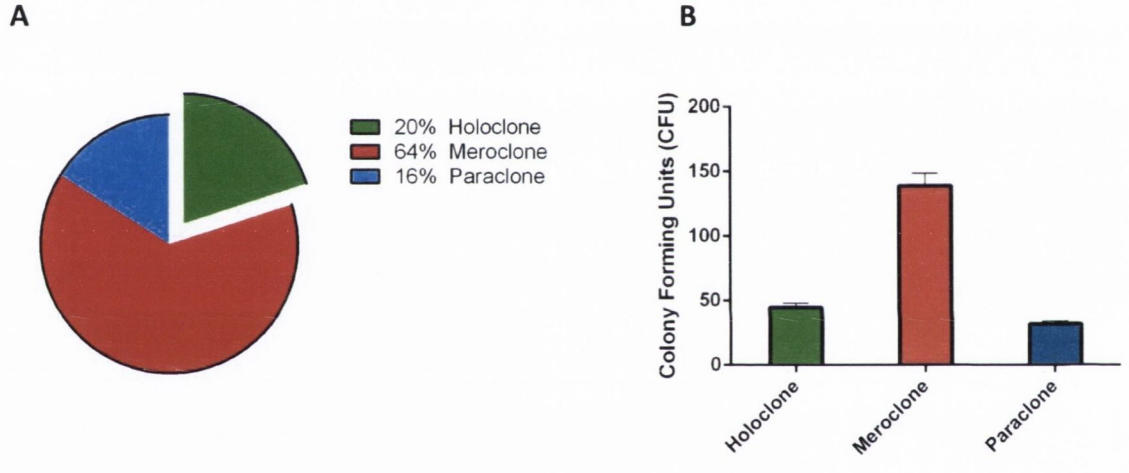

Figure 5.20 *PC-3 colony forming efficiency.*

(A) Percentage of distinct colonies produced by PC-3 cells expressed as a proportion of total colonies produced across multiple experiments (n=3, 15 plates/experiment). (B) Colony forming units of PC-3 cells across multiple experiments (n=3, 15 plates per experiment). Data is graphed as mean and standard error of the mean.

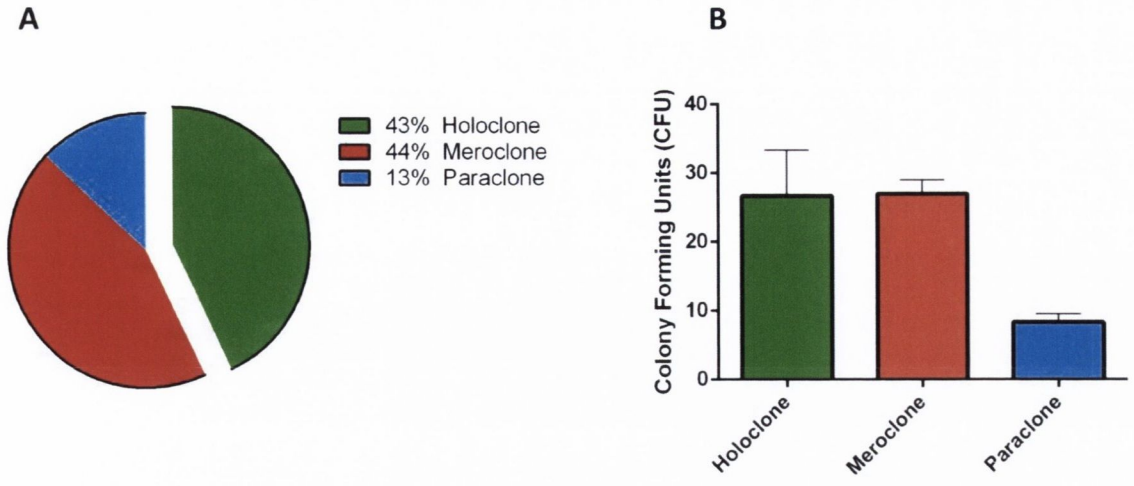

Figure 5.21 *DU145 colony forming efficiency.*

(A) Percentage of distinct colonies produced by DU145 cells expressed as a proportion of total colonies produced across multiple experiments (n=3, 15 plates/experiment). (B) Colony forming units of DU145 cells across multiple experiments (n=3, 15 plates per experiment).

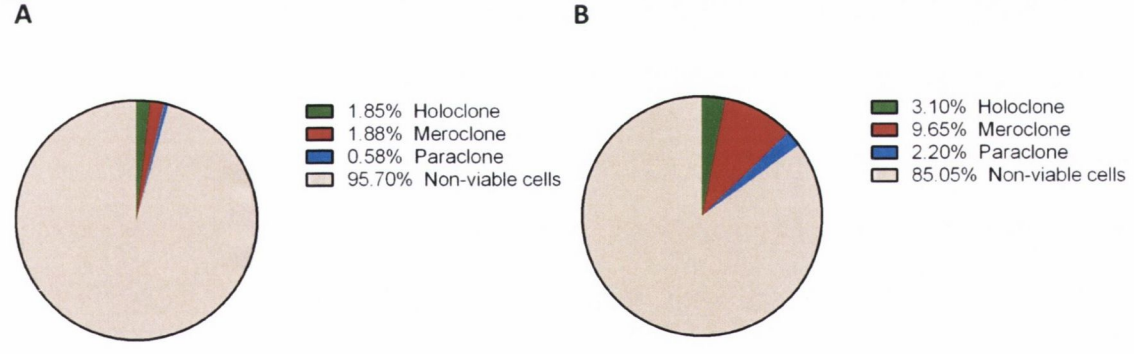

Figure 5.22 *DU145 and PC-3 percentage colonies formed of total cells plated.*

(A) Percentage of each colony generated by DU145 cells expressed as a proportion of total cells plated (n=3, 15 plates/experiment). (B) Percentage of each colony generated by PC-3 cells expressed as a proportion of total cells plated. (n=3, 15 plates/experiment). Note: cells sorted into wells that did not generate colonies are marked as non-viable cells.

5.2.7 Only Holoclones Can Regenerate All Colony Morphologies

Dissociation of distinct PC-3 and DU145 colonies, expansion within a 12-well dish and subsequent resubmission to monoclonal cultivation demonstrated that only holoclones possessed the capability to regenerate all colony morphologies. Meroclones were found to regenerate only meroclones and paraclones, while paraclones exhibited modest ability to regenerate their own morphology. Furthermore, dissociation and replating of PC-3 and DU145 holoclones resulted in a higher frequency of holoclone generation than observed in first-round plating (Figure 5.23, Figure 5.24).

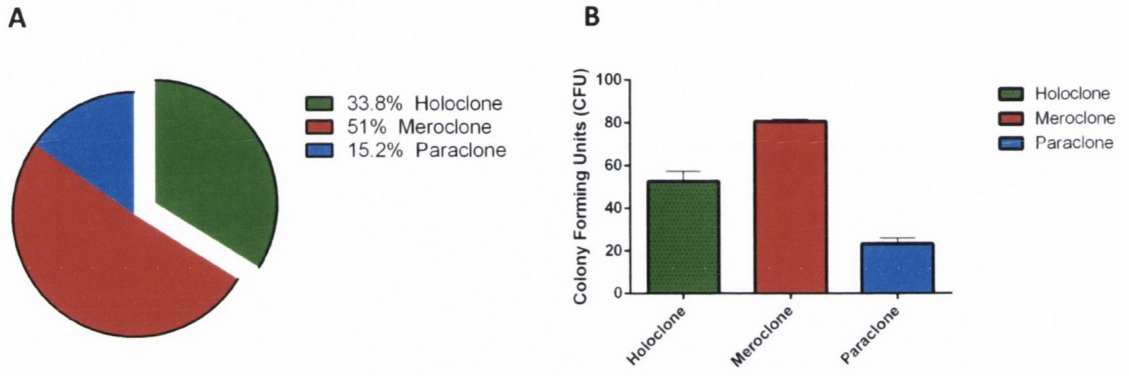

Figure 5.23 *PC-3 colony frequencies yielded from dissociated and replated holoclones.*

Replated holoclones generated a larger proportion of holoclones than normal parental cells. (n=3 experiments, 5 plates/experiment). Data is graphed as mean and standard error of the mean.

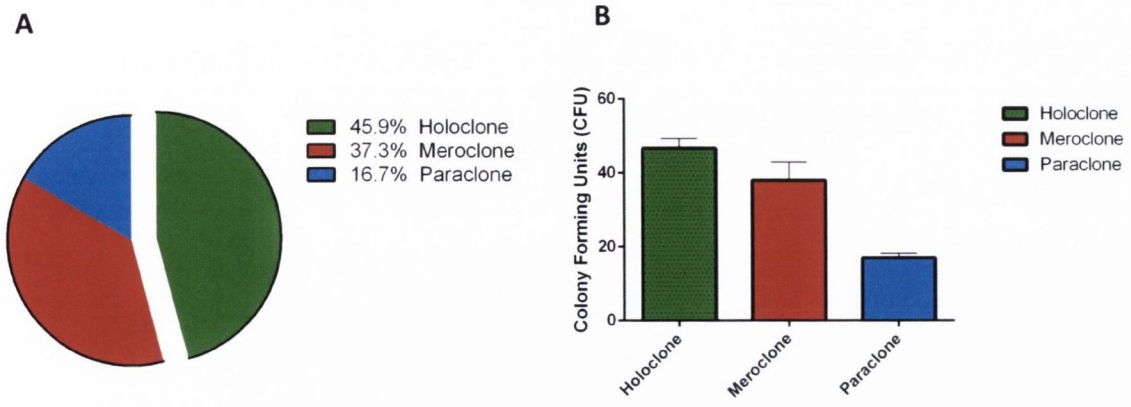

Figure 5.24 *DU145 colony frequencies yielded from dissociated and replated holoclones.*

Replated holoclones generated a larger proportion of holoclones than normal parental cells. (n=3 experiments, 5 plates/experiment). Data is graphed as mean and standard error of the mean.

5.2.8 Holoclones Preferentially Express Stem Cell-Associated Markers

Holoclones originating from PC-3 and DU145 cells were harvested and their putative stem identity was interrogated by examining the expression of a number of critical stem cell associated-markers by quantitative RT-PCR; ALDH1, CD44, CD133, NANOG, POU5F1/OCT4 and SOX2. Expression levels of ALDH1 ($p=0.04$), NANOG ($p=0.001$) and POU5F1/OCT4 ($p=0.04$) were found to be statistically significantly higher in DU145 holoclones than in parental cells (Figure 5.25). Similarly, ALDH1 ($p=0.03$), POU5F1/OCT4 ($p=0.02$) and NANOG ($p=0.04$) were overexpressed in PC-3 holoclones, while CD44, SOX2 and CD133 follow this trend of overexpression in holoclones of both of cell lines (Figure 5.26).

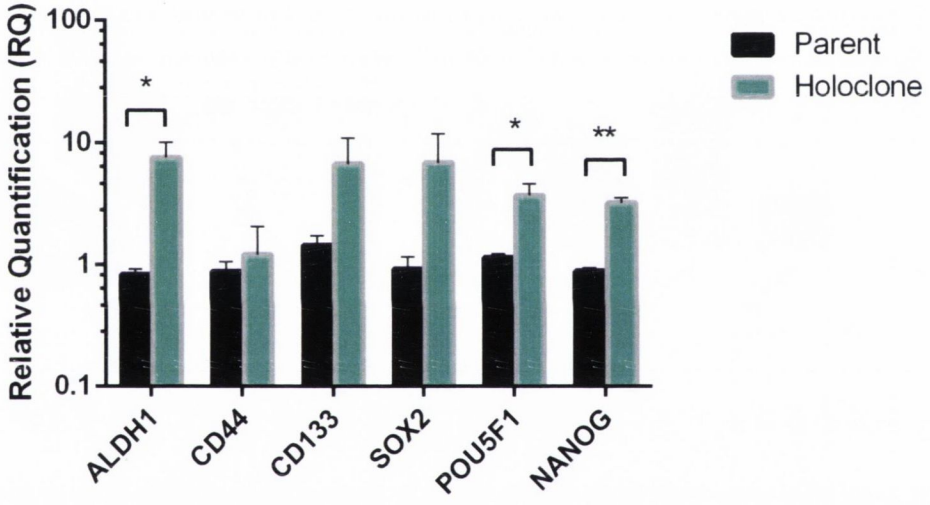

Figure 5.25 *Stemness gene expression analysis in colony forming assay-derived DU145 holoclones.*

ALDH1, POU5F1/OCT4 and NANOG are statistically significantly overexpressed in DU145 holoclones compared to their parental counterpart. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

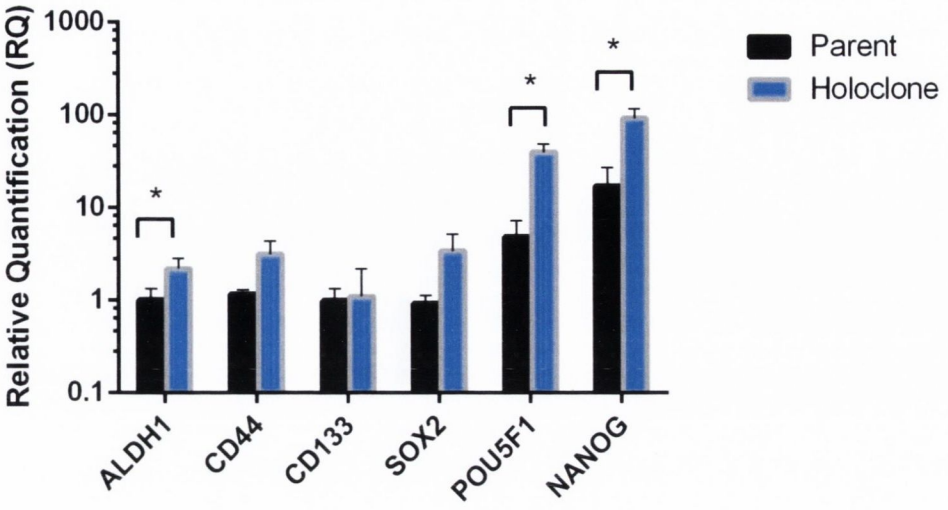

Figure 5.26 *Stemness gene expression analysis in colony forming assay-derived PC-3 holoclones.*

ALDH1, POU5F1/OCT4 and NANOG are statistically significantly overexpressed in PC-3 holoclones compared to their parental counterpart. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

5.3 Discussion

In recent years, a subset of undifferentiated, slow-cycling cells known as cancer stem cells have become the subject of intense research in cancer biology. Malignant stem cells coexist with their highly-proliferating, differentiated progeny within the bulk tumour and possess a self-renewal potential capable of sustaining tumour growth (Jordan *et al.,* 2006). It has been postulated that under the correct conditions, cancer stem cells can mediate treatment resistance in prostate cancer; both to chemotherapy and androgen withdrawal (Wicha *et al.,* 2012). These observations make cancer stem cells a highly desirable target for therapeutic interventions. However in order to explore the development of novel therapeutics, which target cancer stemness, there must first exist a reliable method to isolate and study prostate cancer stem cells *in vitro.* To this end, a number of methods of prostate cancer stem cell isolation were employed in order to explore the constraints and relative adequacy of these approaches. Furthermore, quantitative RT-PCR was performed to examine the expression of a panel of frequently used stem-associated markers in our putative prostate cancer stem cells.

5.3.1 Flow Cytometric Analysis of Putative Stem Fraction

Prostate cancer cell lines are capable of unlimited proliferation *in vitro,* however it remains to be definitively established whether these long-term cultured cell lines retain the cancer stem cell population which has been identified in primary tumours and whether they can be characterised by the same markers of stem potential. For example, the expression of one such marker, CD133 has been shown to be so low in human prostate epithelial cell lines that it cannot be detected by Western blot analysis despite mass culturing of cells (Vander Griend *et al.,* 2008). However, as in haematopoietic lineages and a variety of tissue types, putative stem cells have been isolated from solid human tumours by the measurement of a set of cell surface markers through highly-sensitive multiparametric flow cytometry. One such panel of lineage markers which, has proven fruitful in isolating stem-like cells from prostate malignancies is $CD44^{+}$/integrin$\alpha_2\beta_1^{hi}$/$CD133^{+}$ (Collins *et al.,* 2005). Examination of this antigenic phenotype in our cell line panel has demonstrated that a moderate proportion (ranging from 2.05% in DU145 cells to 16.9% in LNCaP cells) of the population coexpress these markers and as such are enriched for putative stem characteristics. It has been widely reported that only a small fraction of cells (approximately 0.1%) within a tumour possess stem characteristics such as indefinite self-renewal. Based on this expression profile, our results indicate the presence of a larger stem fraction in immortalised cell lines.

There are a number of reasons as to why this might be the case. Firstly, the biological nature of cancer cells may be altered *in vitro* while being passed through generations, perhaps

creating a bias for $CD133^+$ cells which, concomitantly marginally increases the stem population. From a technical standpoint, studies have shown that certain cell surface markers are susceptible to enzymatic digestion, which is a necessity when examining adherent cell lines (Wang *et al.,* 2010). Thus, the choice of protease for cell detachment can influence the antigenicity of cell surface markers (Greve *et al.,* 2012). In particular, trypsin-EDTA, which is widely employed for proteolysis in adherent cell culture, has been shown to degrade surface antigens and cause cellular toxicity resulting in compromised physical properties when subjected to flow sorting (Greve *et al.,* 2012).

Furthermore, previous attempts to isolate and culture highly tumorigenic $CD133^+$ cells from the LNCaP cell line, have demonstrated that despite being cultured from a >98% pure population of $CD133^+$ cells, only 6.15% of $CD133^+$ cells remained following two weeks of culture, indicating that *in vitro* expansion of this population will result in differentiation and a consequent loss of the stem phenotype. This was also observed in a study by Wei *et al.,* (2007) in which a $CD44^+$/integrin$\alpha_2\beta_1^{hi}$/$CD133^+$ population was isolated from DU145 cells. Upon culturing this population in medium containing 10% FBS, the majority of cells differentiated as corroborated by an increase in cytokeratin 18 (CK18) expression. Overall, while the limitations associated with the isolation and subsequent expansion of a $CD44^+$/integrin$\alpha_2\beta_1^{hi}$/$CD133^+$ population would appear to preclude its successful application to the large-scale analysis of prostate cancer stem cells *in vitro*, it does provide an insight into the proportion of cells within established cell lines, which potentially retain stem characteristics despite long-term culture.

5.3.2 High Salt Agar Holoclones Express Stem-associated Genes

Previous work within our laboratory has optimised a low-density cell culture technique, which preferentially generates cancer cell holoclones (Sommerville *et al., in preparation*). This method involves culturing cells on a non-adherent high-salt agar surface. The high concentration of salt is postulated to create a harsh microenvironment in which only putative quiescent stem cells can survive. This technique was applied to our cell line panel and morphologically homogeneous holoclones were generated for each of the four cell lines (Figure 5.6, Figure 5.7, Figure 5.8, Figure 5.9). Each cell line exhibited differential efficiencies to generate holoclones, with DU145 cells exhibiting the most protracted incubation at approximately 6 weeks. It is worthy to note that holoclone generation using this technique was a highly unreliable and lengthy process.

The stem potential of these colonies was assessed by examining the expression of a panel of stem cell-associated markers comprising previously identified prostate cancer-specific markers including ALDH1, CD133, Integrin$\alpha2\beta1$, and CD44 and the critical embryonic

pluripotency markers POU5F1/OCT4 and NANOG. While a number of stemness genes (NANOG, POU5F1/OCT4, CD44) were found to be significantly enriched in holoclones propagated through the high salt agar technique compared to their normal parental counterparts, the level of expression of each marker was found to be highly variable between replicates (Table 5.2). These results indicate that this technique may not enrich for a pure stem cell population. Holoclones generated in this manner may represent a mixed population of stem and late progenitor cells. Furthermore, the expression patterns of these stem-associated genes were found to vary greatly between cell lines, which again would question the efficacy of this technique to generate true cancer stem cells.

However, NANOG was found to be significantly upregulated in holoclones derived from the PC-3 and 22Rv1 cell lines. NANOG, OCT4/POU5F1 and SOX2 are members of a critical group of transcription factors responsible for the maintenance of pluripotency and self-renewal in embryonic stem cells (Mitsui *et al.,* 2003). NANOG has been implicated as the principal regulator of pluripotency in human embryonic development as small-interfering RNA (siRNA)-mediated downregulation of this gene in embryonic stem cells has been shown to induce differentiation (Hyslop *et al.,* 2005). NANOG expression has been detected in multiple solid tumour types and its elevated expression is believed to predispose a poor prognosis particularly in breast and colorectal cancers (Nagata *et al.,* 2012; Meng *et al.,* 2010). Furthermore, functional studies have demonstrated that NANOG overexpression is capable of inducing a cancer stem cell phenotype in several cancers. For example, Jeter *et al.,* (2011) demonstrated that ectopic expression of NANOG in DU145 and LNCaP cells resulted in enhanced clonogenicity and tumour regenerative ability. These results are highly suggestive of a mechanistic link between NANOG overexpression and tumour development/progression. It is prudent to note that Jeter *et al.,* (2011) also reported a concomitant upregulation of CD133 and ALDH1 as a result of lentiviral promoter-driven NANOG expression. Our results contradict these findings, infact both PC-3 and 22Rv1 holoclones, which exhibited the most significant upregulation in NANOG expression, also displayed a downregulation or complete loss of ALDH1 and CD133. While, this observation may be owing to a feature of these particular cell lines, these results may also indicate that high-salt agar holoclone derivation does not select for a pure stem population. Overall, these findings indicate a definitive trend toward upregulation of stem associated-genes in salt agar-derived holoclones, particularly those of the PC-3 and DU145 cells, however the ambiguity surrounding the ontogeny of these holoclones coupled with the relative inefficiency of this technique indicate that it is not an effective model for the isolation and characterisation of prostate cancer stem cells.

5.3.3 Growth of PC-3 and DU145 Cells in Stem Cell Medium did not Induce Sphere Formation

Sphere culture has been employed in previous studies to isolate putative prostate cancer stem cells (Duhagon *et al.,* 2010; Rybak *et al.,* 2013). This technique involves the growth of cells in serum-free medium supplemented with epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin. This technique is based upon the assumption that only putative cancer stem cells can survive in the absence of serum. We have demonstrated that the culture of PC-3 and DU145 cells in stem cell medium did not induce the formation of three-dimensional prostatospheres. However, a recent study by Deep *et al.,* (2014) has demonstrated the efficacy of performing the prostatosphere assay on ultra-low attachment plates. Much like survival in the absence of serum, it is postulated that only stem-like cells can survive in low-adherence conditions. The ability of this form of culture to enrich for prostate cancer stem cells has also been demonstrated by Wang *et al.,* (2013) who employed ultra-low adherence culture in stem cell medium to isolate prostate cancer stem cells from primary prostate cancer cultures. Thus, it may be possible that seeding of DU145 and PC-3 cells in specialised low-adherence plates would induce a more definitive adoption of spheroid morphology; however gene expression profiling has confirmed that growth in serum-free stem cell medium induces a stem genotype, particularly in the case of DU145 cells. POU5F1/OCT4, NANOG and SOX2 were all found to be significantly upregulated in 'sphere cells' when compared to the resting parental counterpart (Figure 5.14). Furthermore, while they did not reach significance both CD44 and CD133 displayed upregulation. All of the stem-associated markers were found to be upregulated in PC-3 cells cultured in this medium; however their expression did not reach significance (Figure 5.15).

It is noteworthy that DU145 cells grown in stem cell medium displayed a statistically significant upregulation of SOX2 ($p= 0.001$). These findings closely parallel previous work performed by Rybak *et al.,* (2013) in which they hypothesise that SOX2 expression is positively regulated by the activation of epidermal growth factor receptor (EGFR) signalling through addition of exogenous epidermal growth factor (EGF). In support of this notion, they have demonstrated that blocking EGFR activation in putative prostate cancer stem cells results in a reduction in SOX2 expression and diminished self-renewal activity. Furthermore, ectopic SOX2 expression alone is not sufficient to enhance DU145 sphere self-renewal in the absence of EGFR signalling, suggesting the involvement of SOX2 in EGFR-dependent self-renewal, however unravelling the relationship which exists between SOX2 and EGFR signalling will require further investigation.

5.3.4 Prostate Cancer Cell Lines Generate Morphologically Heterogeneous Colonies in Clonal Culture

As discussed in section 5.1.5, the extension of colony forming assay to immortalised cancer cell lines has seen the identification of putative cancer stem cells in multiple cancer types. This technique generates phenotypically plastic colonies with hierarchical proliferative abilities believed to parallel stem, transit-amplifying and differentiated cells. In order to assess the efficacy of this technique in deriving prostate cancer holoclones, we applied this method to our cell line panel. Both 22Rv1 and LNCaP cells were unable to generate colonies and as a result were removed from further rounds of experiments. DU145 and PC-3 cells successfully generated morphologically heterogeneous holoclones, meroclones and paraclones (Figure 5.16, Figure 5.17, Figure 5.18). These colonies were identified based upon their differential morphological features and closely resembled those described in previous studies (Tan *et al.*, 2011). PC-3 and DU145 cells were found to exhibit differential propensities to generate each colony type. Examination of colony composition figures across multiple experiments (n=3) has demonstrated that of the total colonies produced by PC-3 cells approximately 20% were holoclones (which equates to roughly 50 holoclones). While a larger percentage of the total colonies produced by DU145 cells were holoclones (43%), this equates to fewer colonies overall. Interestingly, when these figures are correlated to the total number of cells plated it becomes apparent that only a small proportion of the cellular population is capable of generating holoclones. Approximately 1.85% of DU145 cells and 3.05% of PC-3 cells can generate holoclones. These findings are in keeping with previous reports, which indicate that the stem cell niche can comprise approximately 1-5 % of the malignant population (Collins *et al.*, 2005). These figures also closely parallel the percentage putative stem population residing in each cell line identified through flow cytometric analysis (5.2.1).

To assess the relative self-renewal capacity of each clonal morphology, holoclones, meroclones and paraclones were harvested, dissociated and resubmitted to monoclonal cultivation. Holoclones were the only colony capable of regenerating all three clonal morphologies. Meroclones and paraclones exhibited modest ability to generate their own morphology, indicating that holoclones preferentially possess the ability to self-renew. Furthermore, second-phase plating revealed that dissociated holoclone cells generate a higher frequency of secondary holoclones than do naive parental cells, which indicates the powerful proliferative capacity present within holoclones (Figure 5.23, Figure 5.24). 33.8% of the colonies produced by PC-3 holoclone cells were comprised of secondary holoclones, which equated to approximately 50 colonies, while 45.9% of the colonies produced by DU145 holoclone cells were comprised of holoclones (approximately 45 colonies). These

results are indicative of the maintenance of robust hierarchical stem cell patterns in long-term cultured epithelial cell lines.

5.3.5 Clonally-derived Holoclones Significantly Express Stem Markers

The stem phenotype of clonally-cultivated holoclones was assessed by examining the expression of stem cell-associated genes. Both PC-3 and DU145 holoclones exhibited a significant overexpression of NANOG, OCT4 and ALDH1 while the remaining markers demonstrated a definitive trend toward upregulation.

The seminal paper by Takahashi *et al.,* (2007) which described the reprogramming of adult somatic cells to induced pluripotent stem cells has provided compelling evidence to suggest that differentiated cells maintain the capacity for dedifferentiation (Takahashi *et al.,* 2007). As previously discussed, NANOG has been widely implicated as the gatekeeper of pluripotency; however a recent study by Kumar *et al.,* (2012) has demonstrated that OCT4/POU5F1 can initiate dedifferentiation of melanoma cells to immature stem-like cells. These dedifferentiated melanoma cells exhibited increased resistance to chemotherapeutic agents and developed the ability to form tumour spheres. Furthermore, RNAi-mediated knockdown of OCT4 abrogated this observed cancer stem cell phenotype. This would indicate that OCT4 alone is sufficient to induce lineage reprogramming. Taking these results into consideration, our findings suggest that the cancer stem cell phenotype of clonally-derived holoclones may be acquired through dynamic dedifferentiation mediated by increased OCT4 expression. Further studies have indicated that malignant cells are much more susceptible to OCT4-mediated dedifferentiation than normal cells (Hochedlinger *et al.,* 2005). Kumar *et al.,* (2012) have demonstrated induced OCT4 expression as a response to hypoxia, indicating that endogenous OCT4 expression may be environmentally regulated. This would provide an attractive mechanism to explain the generation of phenotypically plastic colonies through monoclonal cultivation. OCT4 expression may be triggered as part of a survival response to isolated cultivation, however only a small percentage of the cellular population may be capable of responding in this way and thus undergoing dedifferentiation. While this appears a feasible hypothesis, further experimentation is required in order to elucidate the mechanism responsible for cancer stem cell derivation.

5.4 Conclusion

The efficacy of using established cancer cell lines as an *in vitro* model for human cancer has been questioned in a number of studies (Weiss *et al.,* 2000; Burdall *et al.,* 2003). A major disadvantage of cell lines is their relative homogeneity and resultant inadequate representation of tumour behaviour. Indeed, adaptation to *in vitro* culture conditions is

hypothesised to eliminate certain cell types present in the original tumour environment. Furthermore, cell lines in culture are not subject to the same *in vivo* endogenous regulatory influences and many posit that their susceptibility to genotypic and phenotypic drift confounds their application to *in vitro* stem cell studies. However, seminal work by Barrandon and Green (1987) has demonstrated that certain aspects of stem cell growth patterns are intrinsic to epithelial cells. This has led to the hypothesis that key stem cell properties such as asymmetric division are maintained in cells isolated and grown *in vitro*. Thus the morphologic heterogeneity characteristic of malignant cells, which had previously been attributed to genetic instability may infact represent a vestige of normal epithelial stem cell patterns. The fundamental aims of this chapter were to establish whether the intrinsic stem cell hierarchies present within the normal epithelial compartment and within solid tumours, are maintained in long term cultured malignant cell lines and to identify a robust and reliable method for the identification and isolation of putative prostate cancer stem cells. In order to address this question, the feasibility of a number of methods of cancer stem cell isolation was explored, namely flow cytometric sorting, prostatosphere generation, high-salt agar assay and colony forming assay. We have identified the colony forming assay as the most reliable and effectual method for the isolation of putative stem-like cells from malignant prostate cell lines. Holoclones generated in this manner, stably express established stem cell markers and exhibit a profound proliferative and self-renewal capacity. As a result, we decided to take this particular technique forward in our attempts to further characterise the prostate cancer stem cell population.

Xenotransplantation of Prostate Cancer Holoclones

Chapter 6

Chapter 6. Xenotransplantation of Prostate Cancer Holoclones

6.1 Introduction

6.1.1 Xenotransplantation as a Criterion for Defining Cancer Stem Cells

As recently as ten years ago, the cancer stem cell hypothesis was rejected by certain groups of cancer biologists, who postulated that the majority of cells within a tumour possessed a similar propensity to divide and metastasise. However, as previously mentioned, seminal work by Bonnet *et al.,* (1997) provided the first piece of compelling evidence in support of the existence of a cancer stem cell fraction in haematological malignancies. This putative malignant stem cell fraction, displayed surface characteristics complementary to their normal haematopoietic counterpart and only these cells were capable of recapitulating acute myeloid leukaemia in immunodeficient mice. Thus, the capacity to regenerate tumour pathophysiology upon xenotransplantation is now viewed as an essential benchmark in defining cancer stem cells. Despite the technical challenges which have hindered the isolation of cancer stem cells from solid tumours (as discussed in Chapter 5), these findings have been replicated in breast, brain, colon and head and neck cancers (Al Hajj *et al.,* 2003; Galli *et al.,* 2004; O' Brien *et al.,* 2007; Prince *et al.,* 2007). The putative stem fractions, identified by a distinctive surface antigenic profile, were all found to possess greater tumorigenic potential when transplanted into immunosuppressed mice than the cell population in its entirety. Furthermore, cancer stem cell fractions were found to generate tumours which recapitulated the phenotypic heterogeneity of the parent tumour providing further support for the capacity of cancer stem cells to orchestrate multilineage reconstitution.

However, it is noteworthy that there exists considerable controversy surrounding the isolation of cancer stem cells from dissociated solid human tumours (Zhang *et al.,* 2010). As previously mentioned, the existence of prostate cancer stem cells was first described by Collins *et al.,* (2005) when they reported the isolation of a highly clonogenic population of cells from primary human prostate tumours based upon the antigenic phenotype $CD44^{+}$/integrin$\alpha_2\beta_1^{high}$/$CD133^{+}$. This cellular population was found to possess a significant self-renewal and proliferative capacity *in vitro;* however, the *in vivo* tumour-initiating capacity of these stem isolates was not assessed and it has since been suggested that these cells may derive from a normal as opposed to malignant origin. The immortalised cells used in their study were established from primary tumours in low Ca^{2+} serum-free medium, which has been postulated to select against tumour cells permitting the expansion of normal

epithelial basal-derived cells (van Bokhoven *et al.,* 2003). Furthermore, it has been suggested that the predilection of tumours to recruit various types of host cells, including normal progenitor cells results in the isolation of a contaminated cancer stem cell fraction (Ganss *et al.,* 2006; Klassen *et al.*, 2007). Indeed, a number of studies which have claimed to establish human cancer cells in suspension culture have since been regarded as inconclusive. Many of the human cancer stem cell isolates are believed to represent technical artefacts, owing to a variety of reasons including the selection of a normal cell population (Masters *et al.,* 2008). Hence, it has been postulated that in order to circumvent these issues a viable prostate cancer stem cell assay for primary tumour isolates should employ a single-cell cloning technique and the malignant origin of cancer stem cell-containing colonies confirmed by xenotransplantation into immunosuppressed mice. However, our results have demonstrated that classical cancer cell lines pose an attractive alternative source of malignant stem cells. As explored in Chapter 5, a major goal of this thesis was to identify a robust and reliable method for the isolation of prostate cancer stem cells from long-term cultured prostate carcinoma cell lines and as an extension of this aim the current study was undertaken to assess the *in vivo* tumorigenicity of putative stem cell-containing holoclones. Furthermore, it was sought to investigate the biological and histological phenotype of cancer stem cell-derived tumours as a well-established relationship exists between cancer stem cells and tumour perpetuation, local invasion, and the development of distant metastases (Sampieri *et al.,* 2012).

6.1.2 Cancer Stem Cells and Metastasis

There remains a paucity of evidence to conclusively confirm a causal relationship between cancer stem cells and distant metastases, however many pieces of evidence suggest a definite correlation between the cancer stem cell population within a primary tumour and the increased incidence of metastasis. For example, the first identification of solid tumour cancer stem cells was made in metastatic breast cancer lesions, indicating that distant metastases may be fortified with cancer stem-like cells (Al-Hajj *et al.,* 2003). Furthermore, the $CD44^{+}$ population of stem cells isolated in an early study by Patrawala *et al.,* (2006) were found to be highly metastatic. In addition, an expression profiling study performed by Glinksy *et al.,* (2005) examining a defined subtype of highly aggressive prostate carcinoma specimens identified an 11-gene signature comprised mainly of stem-associated genes including BMI-1. The prognostic power of this gene signature was assessed in several independent therapy outcome sets of clinical specimens across multiple solid tumour types and was found to be a powerful predictor of short interval to disease recurrence and secondary metastasis. These findings are somewhat echoed in a study by Ben-Porath *et al.,* (2008), which reports the preferential overexpression of an embryonic stem cell-like signature in histologically poorly

differentiated solid tumours. This signature was composed of transcriptional activation targets of principal pluripotency regulators NANOG, OCT4 and SOX2 and featured a concomitant repression of polycomb-regulated genes. Furthermore, a study by Balic *et al.,* (2006) has reported that locally disseminated breast cancer cells display the putatively stem $CD44^{+}/CD24^{-/lo}$ antigenic phenotype. As previously explored, the cancer stem cell hypothesis states that cancer stem cells possess a potent proliferative and self-renewal ability, which in theory could be sufficient to foster the development of a secondary colony at a distant site (Jordan *et al.,* 2006). Conversely, differentiated progenitor cells are postulated to lack the proliferative ability necessary to establish distant metastases (Tu *et al.,* 2002). However, the most convincing evidence in support of cancer stem cells representing the cell of origin of metastasis has emerged from transplantation studies. The developmental plasticity of adult stem cells has been demonstrated elegantly in a study by Clarke *et al.,* (2000). They have shown that the injection of neural stem cells from the brains of adult mice into the amniotic cavity of chick embryos resulted in chimeric mouse/chick embryos and successfully generated all germ tissue layers. Strikingly, the murine-derived stem cells were found to compose part of anatomically functional beating hearts identified in the embryos. These findings confirm the belief that adult stem cells are not lineage restricted and infact possess a broad differentiation capacity. Cogent parallels can be drawn between these observations and the metastatic process; normal stem cells possess the capacity to migrate to a diversity of tissues, much like malignant cells metastasising. Furthermore, they are developmentally plastic, giving rise to multiple tissue lineages, much like the histological heterogeneity widely observed within tumours.

Cancer-related mortality is more often a result of metastatic dissemination than the primary tumour itself, thus, a thorough understanding of the metastatic process is central to eliminating cancer (Tu *et al.,* 2002). While there remains a dearth of definitive evidence to conclusively identify cancer stem cells as the cell of origin of metastasis, there is little doubt that the intrinsic migratory phenotype of cancer stem cells contributes to the pathologic dissemination of cancer.

6.1.3 Cancer Stem Cells and Angiogenesis

In recent years it has been proposed that aside from their tumour-promoting and self-renewal capacities, cancer stem cells may also function in tumour vascularity (Zhao *et al.,* 2011). Angiogenesis (blood vessel sprouting from pre-existing vessels) is one of the classical hallmarks of malignancy and there is increasing evidence to suggest that cancer stem cells perpetuate tumour cell growth by promoting angiogenesis (Hanahan *et al.,* 2011). A study by Bao *et al.,* (2006) has demonstrated that $CD133^{+}$ stem cell-like glioma cells

(SCLGCs) are often located in close proximity to vascular supply. Furthermore, when compared to their $CD133^-$ negative counterpart, $CD133^+$ SCLGCs generated tumours with increased levels of vascularity and necrosis. Vascular endothelial growth factor (VEGF) expression was also found to be 10-20-fold upregulated in $CD133^+$ SCLGC-derived tumours and these tumours displayed a significantly increased vascular density as confirmed by staining with the angiogenic marker CD31. Strikingly, treatment with the VEGF-neutralising antibody bevacizumab was found to abrogate tumour growth in stem cell-like glioma cell murine xenografts. Further evidence in support of the role of cancer stem cells in angiogenesis has come from a study by Yang *et al.,* (2010). This study demonstrated that the expression levels of several angiogenic factors including VEGF and platelet-derived endothelial cell growth factor (PD-ECGF) are significantly higher in putative high hepatic stem/progenitor (HSC/HPC) profile groups of patients with hepatocellular carcinoma. High expression levels of these HSC/HPC biomarkers relating to angiogenesis and microvessel density was found to be a strong predictor of poor prognosis, indicating that both stem-associated and angiogenic factors may provide novel biomarkers for clinical prediction.

A recent study has identified reputed cancer stem cells from the prostate cancer cell line PC-3, whose derivative xenograft tumours consistently exhibit drastically increased vascularity, when compared to tumours generated by parental PC-3 cells (Zhang *et al.,* 2010). Expression of the endothelial marker CD31 was found to be significantly increased in holoclone-derived tumours. These results were validated by immunohistochemistry, which clearly demonstrated the large increase in CD31-positive vascular area in these tumours. These findings suggest that prostate cancer stem cells may possess a strong capacity to induce tumour vascularisation and are consistent with the hypothesis that cancer stem cell populations promote angiogenesis by secreting increased levels of pro-angiogenic factors.

There is an abundance of evidence to support the notion that self-renewing cancer stem cells play a central role in malignant processes, including tumour growth, invasion, dissemination, angiogenesis and treatment failure. Achieving a full understanding of how this rare cellular subpopulation coordinates the diversity of pathways involved will surely be commensurate to conquering cancer.

6.1.4 Experimental Hypothesis and Aims

A fundamental aim of this thesis was to establish an unequivocal method for the isolation of prostate cancer stem cells from established cell lines, and to use this as a platform to address some of the major issues which have frustrated previous attempts to characterise this population, including controversy surrounding their ontogeny and isolation based upon ambivalent surface marker expression. Robust tumour formation upon murine

xenotransplantation of putative cancer stem cells has emerged as the gold-standard in substantiating their stem identity (Visvader *et al.*, 2008). To this end, the hypothesis of this work was that our monoclonally-derived holoclones contained a population of true prostate cancer stem cells which could recapitulate tumours upon transplantation into NOD/SCID mice tantamount to the histopathological characteristics of parental tumours.

The primary aims of these investigations were to confirm the stem identity of our PC-3 and DU145 holoclones by transplantation of cells into NOD/SCID mice, to examine the *in vivo* behaviour of prostate cancer stem cells when isolated from the differentiated bulk population, to address previous evidence implicating cancer stem cells in angiogenesis and the development of metastasis and to assess the biological characteristics of derivative tumours by;

- Comparing and contrasting the growth characteristics of holoclone and parental cell-derived tumours; tumour volume and final tumour mass.

- Determining the degree of retention of intrinsic stem potential in holoclone-derived tumours by analysing the expression of a stem-associated gene panel by qRT-PCR; ALDH1, CD44, CD133, OCT4/POU5F1, SOX2 and NANOG.

- Employing immunohistochemistry to examine the expression of the vascular marker CD34 and epithelial mesenchymal transition (EMT)-associated markers in holoclone and parental cell-derived tumours (E-cadherin and vimentin).

6.2 Results

6.2.1 Feasibility Murine Xenotransplantation

6.2.1.1 PC-3 Holoclones Generate Tumours Macroscopically Similar to Parental Cells

In order to assess the *in vivo* tumour-initiating capacity of PC-3 holoclones a feasibility transplantation experiment was performed whereby 1000 PC-3 holoclone cells were subcutaneously injected in matrigel above the right hind-limb of NOD/SCID mice (n=4). As a control 1000 naive PC-3 parental cells were similarly injected into NOD/SCID mice (n=4). The first palpable signs of tumour growth were observed approximately 38 days following implantation. Mice were euthanised individually via CO_2 asphyxiation followed by cervical dislocation to confirm death when the tumours reached the predefined ethical limit of 10 mm x 10 mm (Figure 6.1, Figure 6.2).

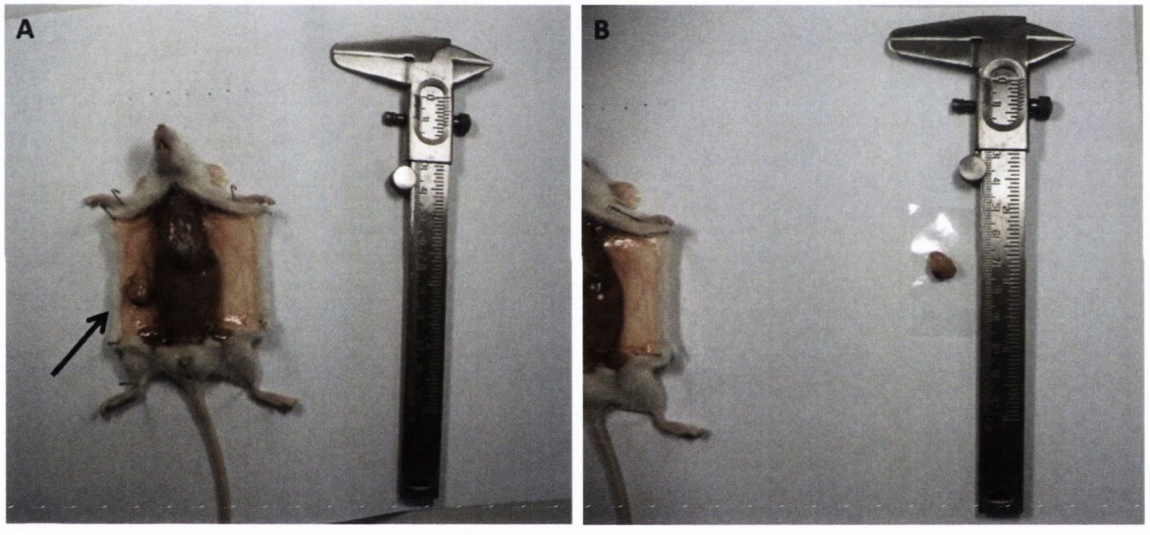

Figure 6.1 *PC-3 parental cell-derived tumour.*

(A) Tumour has been excised from above right hind limb and placed beside body (arrow). All organs appeared healthy with no sign of local or distant metastases. (B) Excised tumour measured ~ 1 cm.

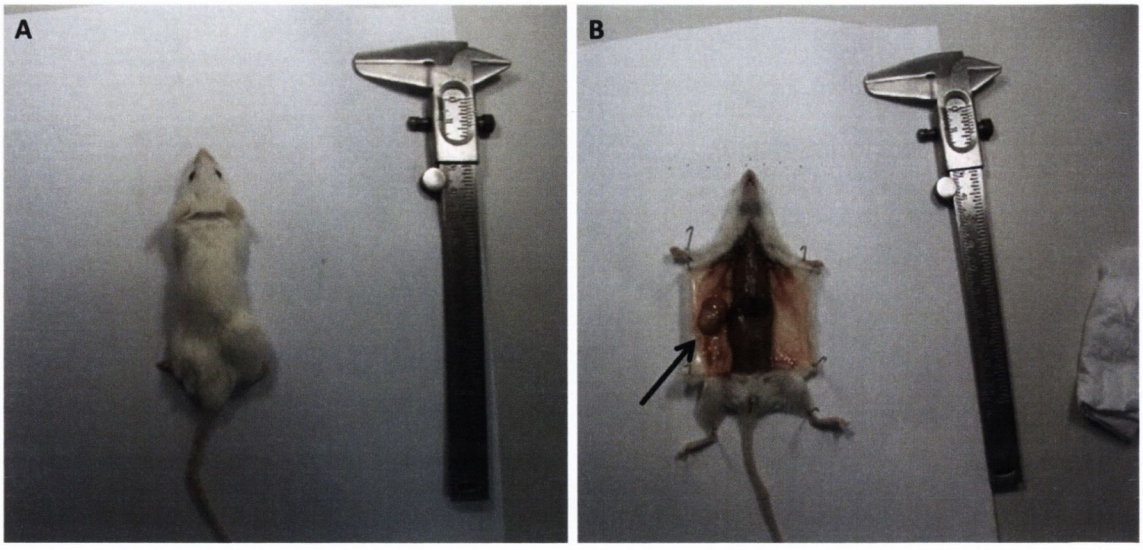

Figure 6.2 *PC-3 holoclone-derived tumour.*

(A) Tumour in-situ can be seen protruding through the fur from above the right hind limb of the mouse. (B) Tumour has been excised and placed beside the body (arrow).

6.2.1.2 PC-3 Holoclone-derived Cells Preferentially Metastasise upon Xenotransplantation

Following euthanisation, dissection of the animals revealed one mouse in the holoclone group possessed a large tumour invading the peritoneal wall in the midsection of the body. The location of this tumour was noted as unusual as they typically developed in close proximity to the injection site beside the hind limb (Figure 6.3). This mouse was found to possess swollen lymph nodes, and distinctive anomalous nodules in the thoracic cavity and the mesentery surrounding the stomach and spleen. The lungs were also found to be small, most likely due to space limitation caused by the thymic nodules. Histopathological examination of these specimens revealed putative metastasis of PC-3 holoclone-derived tumour cells to the spleen and thymus (Figure 6.4).The epithelial nature of these putative metastases was confirmed by immunohistochemistry of the anti-cytokeratin CAM 5.2, which reacts with human cytokeratins 8 and 18 (Figure 6.5). No signs of metastasis were observed in any of the remaining PC-3 parental and holoclone xenografts.

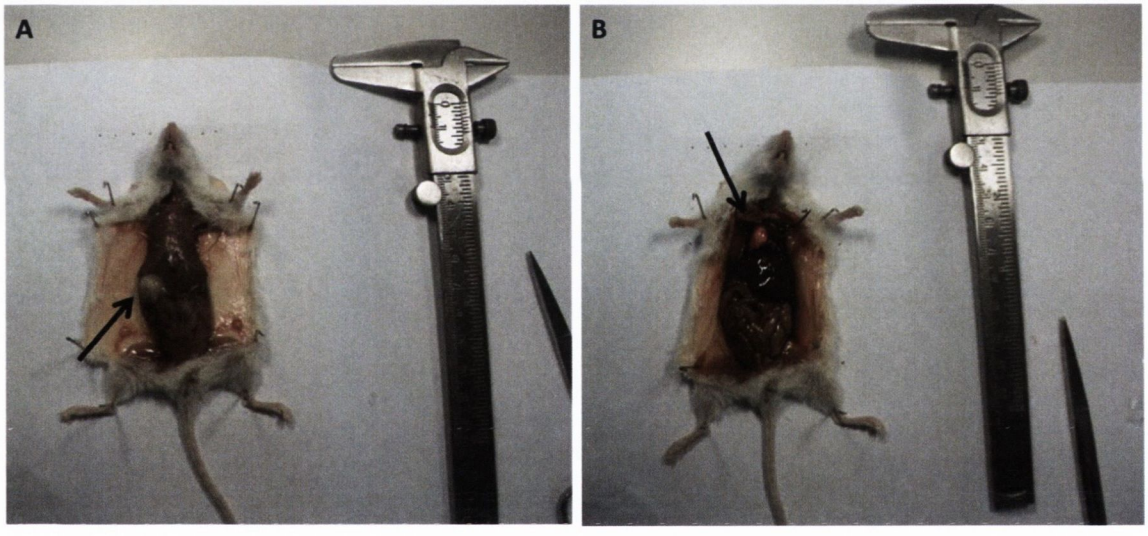

Figure 6.3 *PC-3 holoclone-derived tumour and putative metastases.*

(A) Unlike the rest of the xenograft tumours this tumour was found to grow in an unusual location, into the peritoneal wall away from the subcutaneous injection site (arrow). (B) Upon dissection a white nodule was observed near the thymus. These nodules were also observed in the mesentery near the spleen and stomach (not shown).

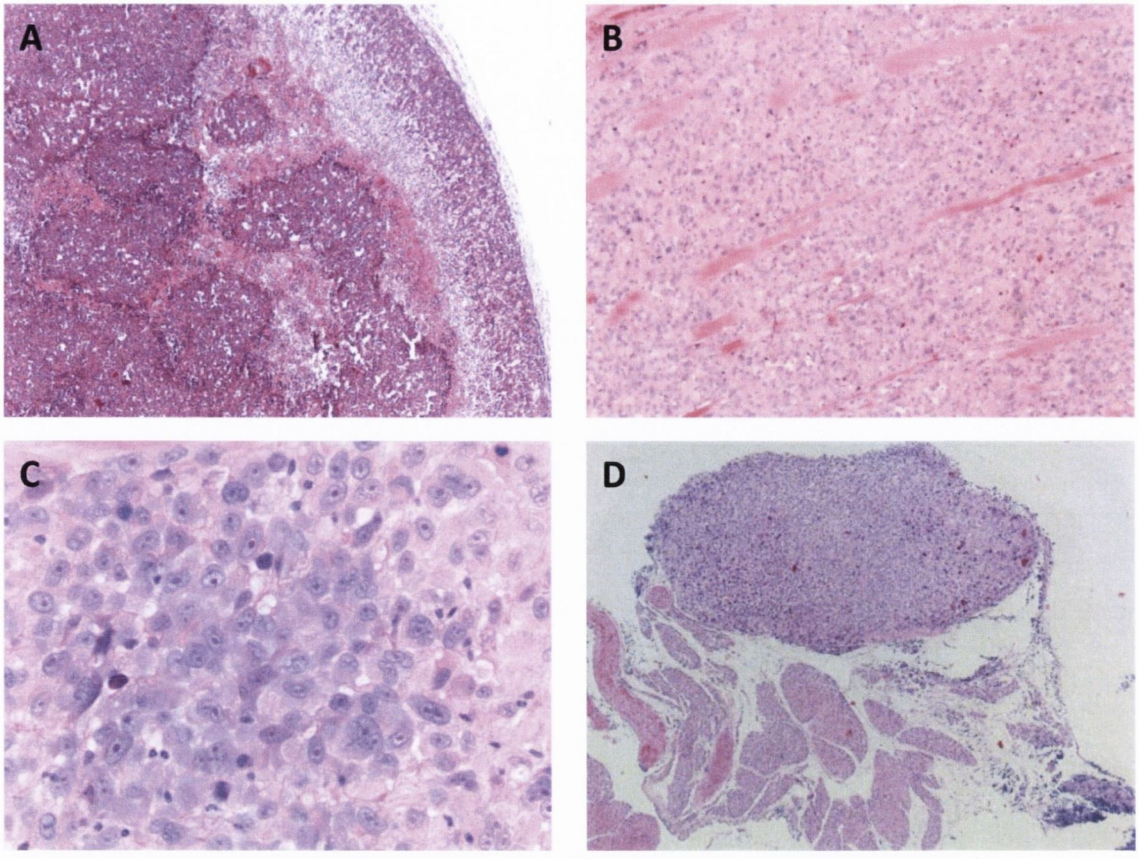

Figure 6.4 *PC-3 parental and holoclone-derived H&E-stained xenograft tumours.*

(A) PC-3 parental tumour (5X) (B) PC-3 holoclone tumour (10X) (C) PC-3 holoclone tumour (40X) (D) PC-3 holoclone metastatic tumour (5X). Histopathological analysis revealed no major histological difference between tumours derived from parental cells and holoclone cells. *magnification refers to objective lens throughout.

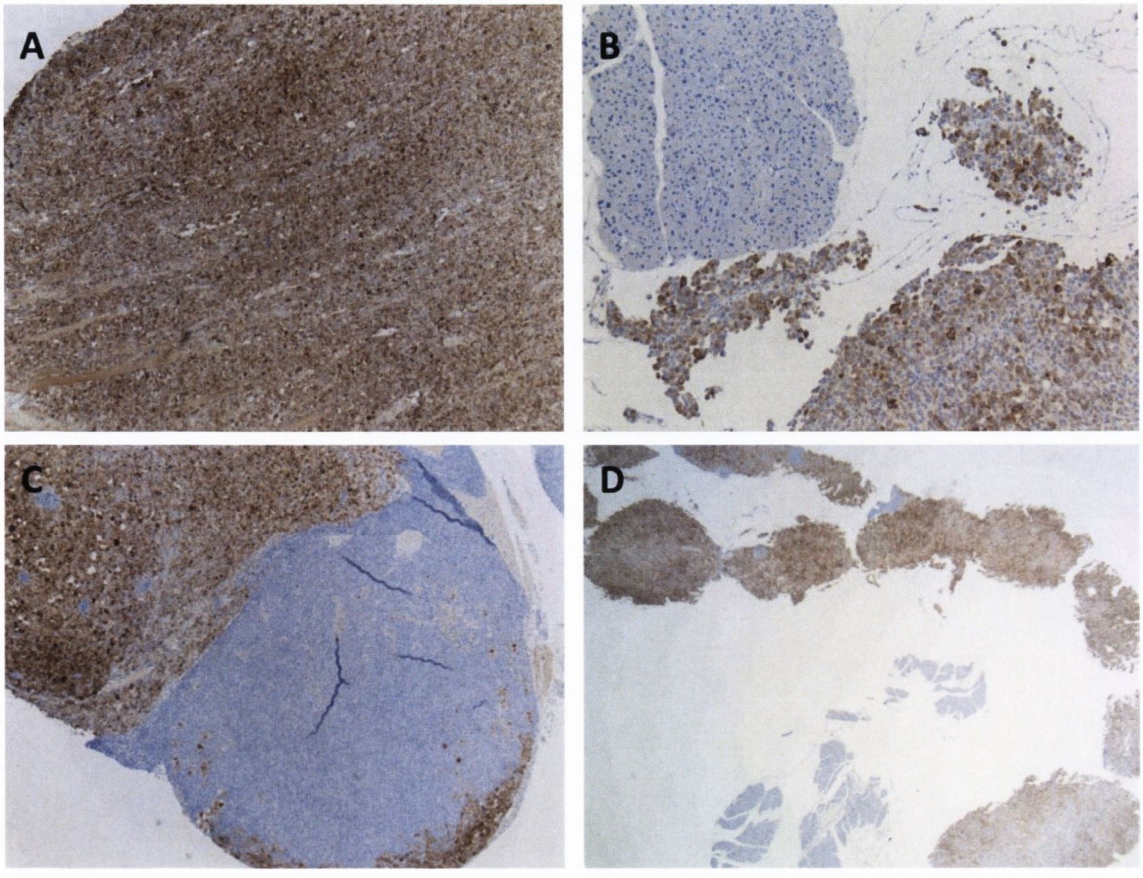

Figure 6.5 *Representative CAM 5.2 staining of PC-3 holoclone-derived primary tumour and putative metastases.*

(A) CAM 5.2-positive staining in PC-3 holoclone-derived primary tumour (5X). (B & C) CAM 5.2-positive staining of PC-3 holoclone-derived thymic metastases (5X). (D) Perisplenic nodules staining positive for CAM 5.2 (5X).

6.2.1.3 Investigation of Stem Profile in PC-3 Parent and Holoclone Xenograft Tumours

Monoclonally-derived PC-3 holoclones were found to significantly overexpress stem-associated genes when compared to their parental counterpart (Chapter 5). In order to determine whether this intrinsic stem profile was maintained in xenograft tumours derived from these holoclones, qRT-PCR was used to analyse the expression of the same panel of stem-associated markers; ALDH1, CD133, CD44, NANOG, POU5F1/OCT4 and SOX2 in formalin-fixed paraffin-embedded holoclone and parental cell-derived tumours. CD133 was not expressed in parental or holoclone-derived tumours. CD44, SOX2, OCT4/POU5F1 and NANOG were all found to be more highly expressed in holoclone-derived tumours when compared to tumour material originating from parental cells, while ALDH1 was more highly expressed in PC-3 parental-derived tumours. Expression levels did not vary widely enough to reach the level of significance.

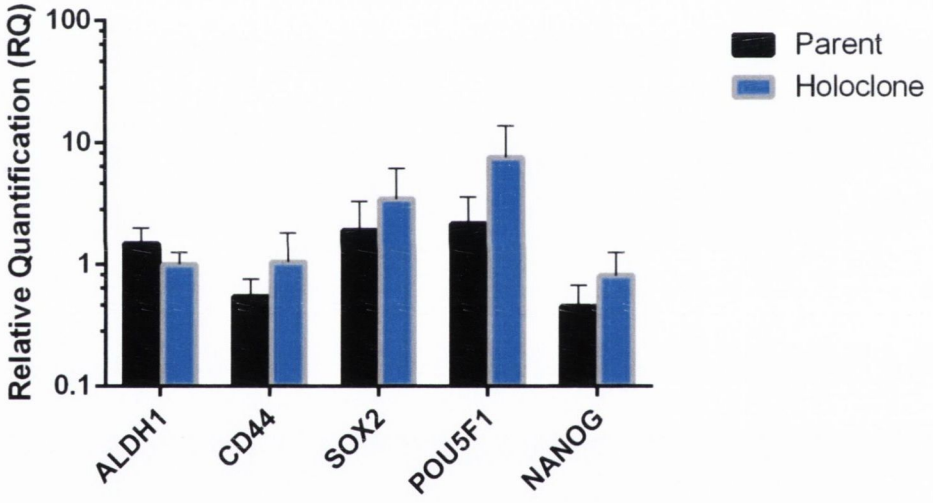

Figure 6.6 *Stemness gene expression analysis in PC-3 parental and holoclone-derived xenograft tumours (n=4).*

CD44, SOX2, NANOG and POU5F1/OCT4 were all found to be more highly expressed in holoclone-derived tumours than those originating from parental PC-3 cells, while ALDH1 was more highly expressed in parental-derived tumours. CD133 was not expressed in parental or holoclone-derived tumours.

6.2.2 Validation Murine Xenotransplantation

6.2.2.1 PC-3 Parent and Holoclone-derived Tumours

In order to further investigate the results stemming from initial feasibility transplantation assays, a further xenotransplantation experiment was performed whereby 3000 PC-3 holoclone cells in matrigel were injected subcutaneously into the flanks of NOD/SCID mice (n=6). As a control, 3000 PC-3 naïve parental cells were similarly injected subcutaneously into the flanks of NOD/SCID mice (n=6). 49 days following injection palpable tumours were noted in both groups. All mice were euthanised via cervical dislocation when one or more tumours reached the predefined ethical limit of 20 x 20 mm (Figure 6.7). Tumours were histologically prepared for pathological examination. Tumours within each group (parent vs. holoclone) were found to possess similar histopathological features. Extensive necrosis was noted within tumours arising from both parental and holoclones cells (Figure 6.8). PC-3 holoclone-derived tumours were found to be markedly pleiomorphic with highly prominent nucleoli. The presence of abundant mitoses was also noted in these tumours.

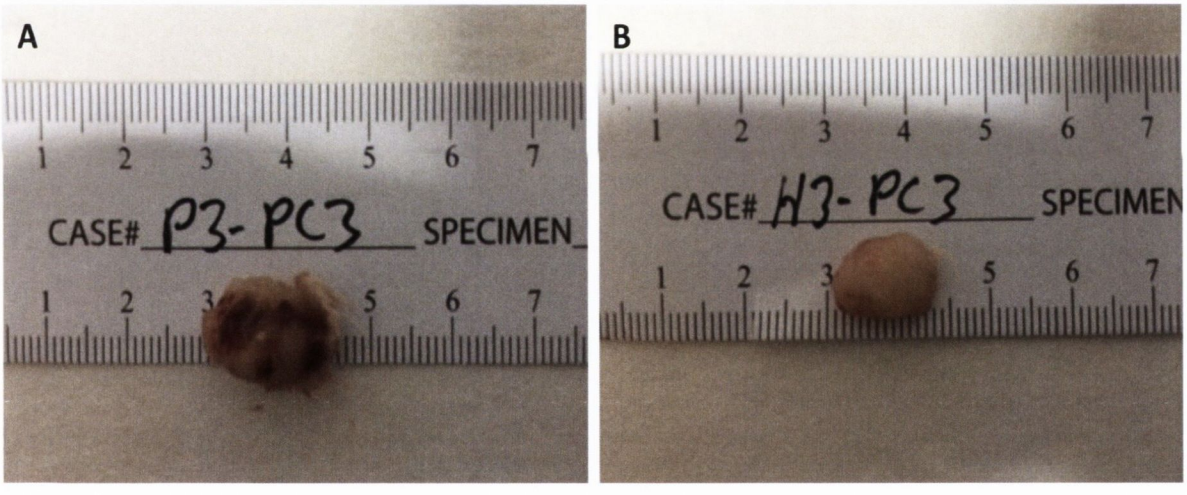

Figure 6.7 *Representative PC-3 parent and holoclone-derived tumour upon excision.*

(A) PC-3 parental cell-derived tumour. (B) PC-3 holoclone-derived tumour.

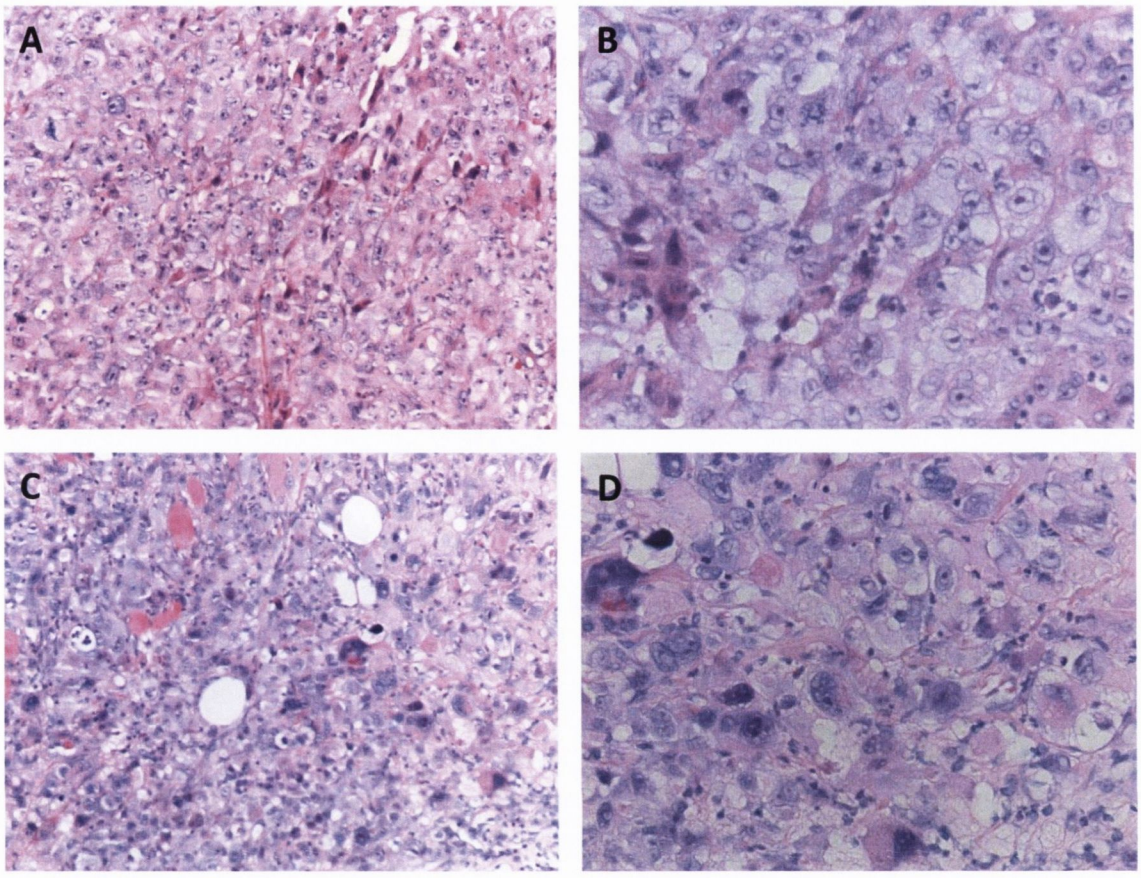

Figure 6.8 *Representative PC-3 parent and holoclone derived H&E-stained tumour sections.*

(A) PC-3 parent-derived tumour (20X). Tumour was found to infiltrate the skin and extensive necrosis was present. (B) PC-3 parent-derived tumour (40X) (C) PC-3 holoclone-derived tumour (20X). Cells were found to be markedly pleiomorphic with very prominent nucleoli. There was abundant mitosis, widespread vascular invasion and focal necrosis present. Tumour was also found to infiltrate the skeletal muscle. (D) PC-3 holoclone-derived tumour (40X).

6.2.2.2 Disparate PC-3 Parent and Holoclone-derived Tumour Dimensions

Tumour dimensions were sequentially measured by external calipers every four to five days following first palpation and tumour volume was calculated by use of the modified ellipsoid formula ½(Length x Width^2) (Euhus *et al.*, 1986). Tumours were harvested one week following the final *in vivo* tumour volume measurement and *ex vivo* dimensions were obtained by measurement with calipers. Tumour mass was also obtained *ex vivo* using a fine balance. While they possessed similar volumes upon initial palpation, tumours derived from PC-3 parental cells were found to be continuously larger than those derived from holoclones (Figure 6.9). No statistically significant difference was identified in *ex vivo* tumour volumes; however it is noteworthy that those derived from parental cells had on average a larger tumour volume (Figure 6.10). Furthermore, PC-3 parental cell-derived tumours were found to weigh more than those generated by holoclones (Figure 6.11).

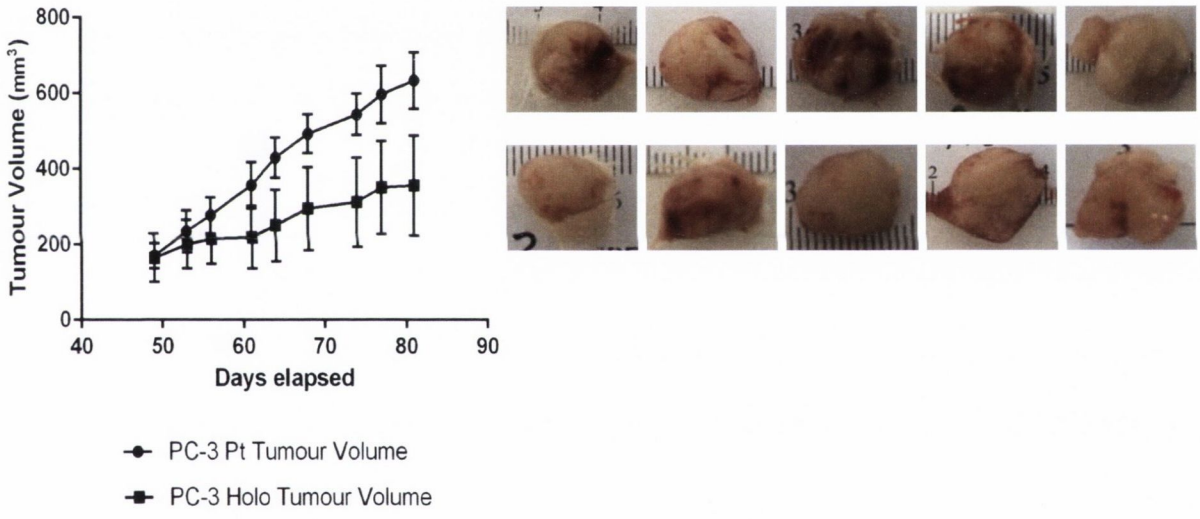

Figure 6.9 *Xenotransplantation growth curve following subcutaneous injection of 3000 PC-3 parental and holoclone cells into NOD/SCID mice (n=6).*

Palpable tumours were noted in each group at 49 days following injection. Growth curves were stopped when one mouse from each group reached the predefined ethical limit of 20 mm x 20 mm palpable tumour. Representative tumours can be seen to the right of the growth curves. Error bars represent standard error of the mean.

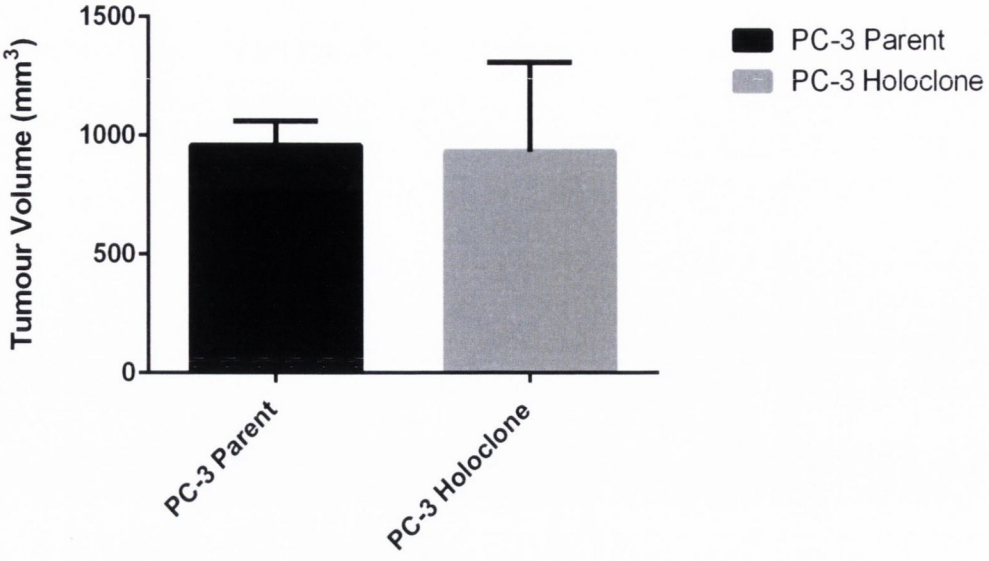

Figure 6.10 *PC-3 parent and holoclone-derived tumour final volume upon excision 87 days following subcutaneous injection (n=6).*

No statistically significant difference in tumour volume was observed between PC-3 parental and holoclone-derived tumours; however more variation was noted in the size of holoclone-derived tumours.

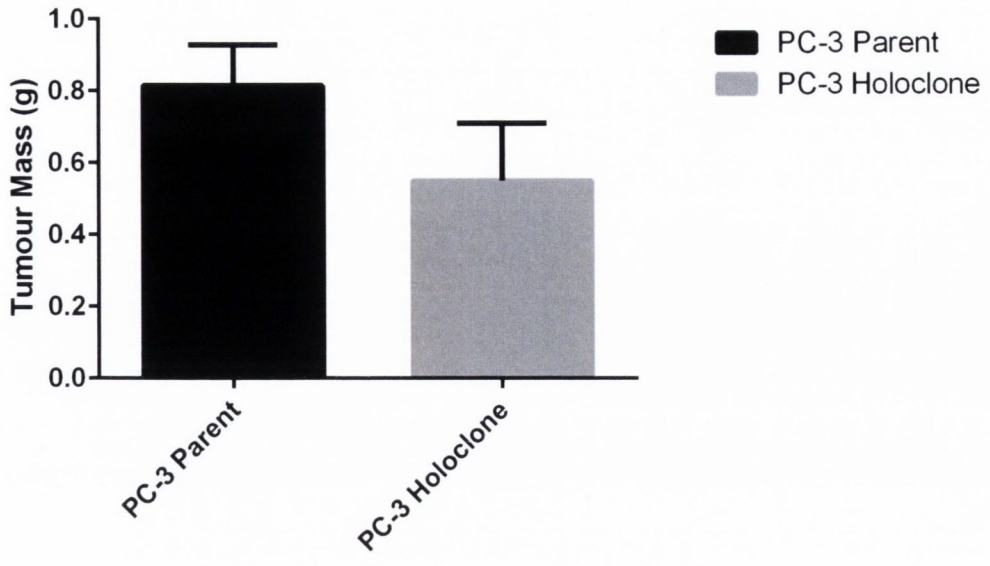

Figure 6.11 *PC-3 parental and holoclone-derived tumour mass upon excision at 87 days following subcutaneous injection (n=6).*

No statistically significant difference in tumour mass was identified between parent and holoclone-derived tumours however PC-3 parental cells had an overall greater mass than holoclone-derived tumours.

6.2.2.3 Investigation of Stem Profile in PC-3 Parent and Holoclone Xenograft Tumours

Results from feasibility xenotransplantation experiments indicated that the stem expression profile characteristic of PC-3 holoclones persisted in their derivative tumours. In order to further investigate these results in the murine validation study, qRT-PCR was used to analyse the expression of the same panel of stem-associated markers; ALDH1, CD133, CD44, NANOG, POU5F1/OCT4 and SOX2 in formalin-fixed paraffin-embedded holoclone and parental cell-derived tumours. Contradictory to previous findings, SOX2, NANOG and POU5F1/OCT were found to be downregulated in holoclone-derived tumours. ALDH1 was found to be overexpressed in holoclone-derived tumours while CD44 was statistically significantly downregulated (p= 0.04) in tumours generated by PC-3 holoclones (Figure 6.12).

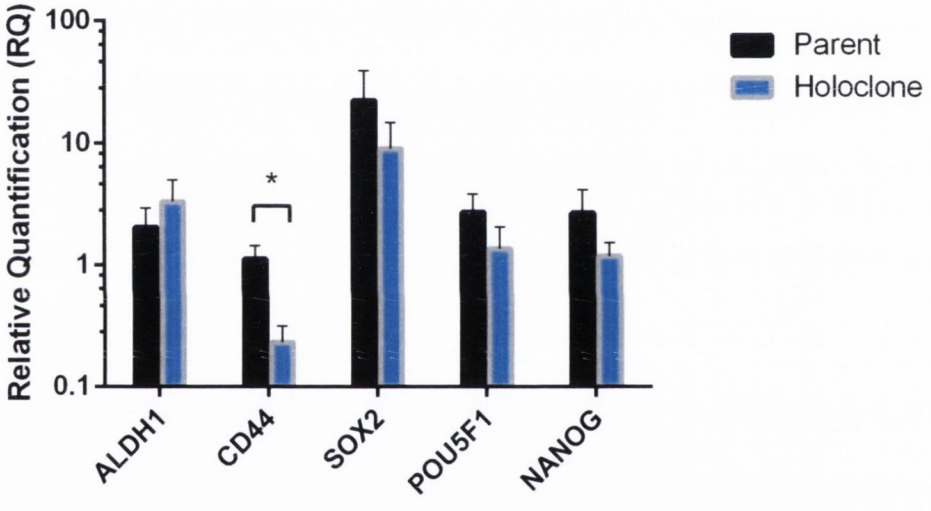

Figure 6.12 *Stemness gene expression analysis in PC-3 parental and holoclone-derived xenograft tumours (n=4).*

ALDH1 was found to be upregulated in holoclone-derived tumours when compared to the parental counterpart, however the remaining stem-associated markers were found to be downregulated in PC-3 holoclone-derived tumours. CD44 in particular was statistically significantly downregulated tumours generated by PC-3 holoclone cells. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

6.2.2.4 DU145 Parent and Holoclone-derived Tumours

In order to assess the *in vivo* tumour forming capacity of DU145 holoclones xenotransplantation assays were performed. 3000 DU145 holoclone cells in matrigel were subcutaneously injected into the flanks of NOD/SCID mice (n=6). As a control 3000 naïve DU145 parental cells were similarly injected into the flanks of NOD/SCID mice (n=6). 49 days following injection, palpable tumours were noted in both groups (Figure 6.13, Figure 6.14). All mice were euthanised via cervical dislocation when one or more tumours reached the predefined ethical limit of 20 x 20 mm. Tumours were histologically prepared for pathological examination. Much like those generated by PC-3 holoclones, DU145 holoclone-derived tumours were histologically similar to parental tumours. However muscle infiltration was noted to be more highly prevalent in holoclone-derived tumours and focal areas of clear cell change were observed (Figure 6.15).

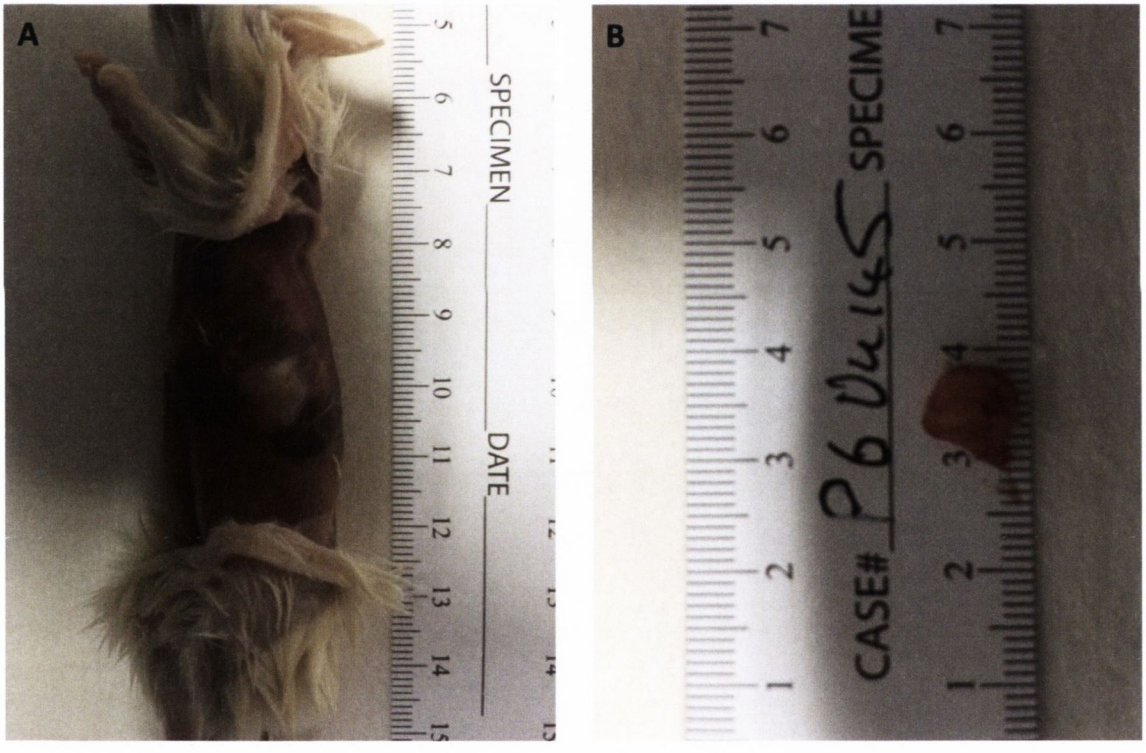

Figure 6.13 *DU145 parent-derived tumour.*

(A) Parental cell-derived tumour in situ. (B) DU145 parental tumour upon excision.

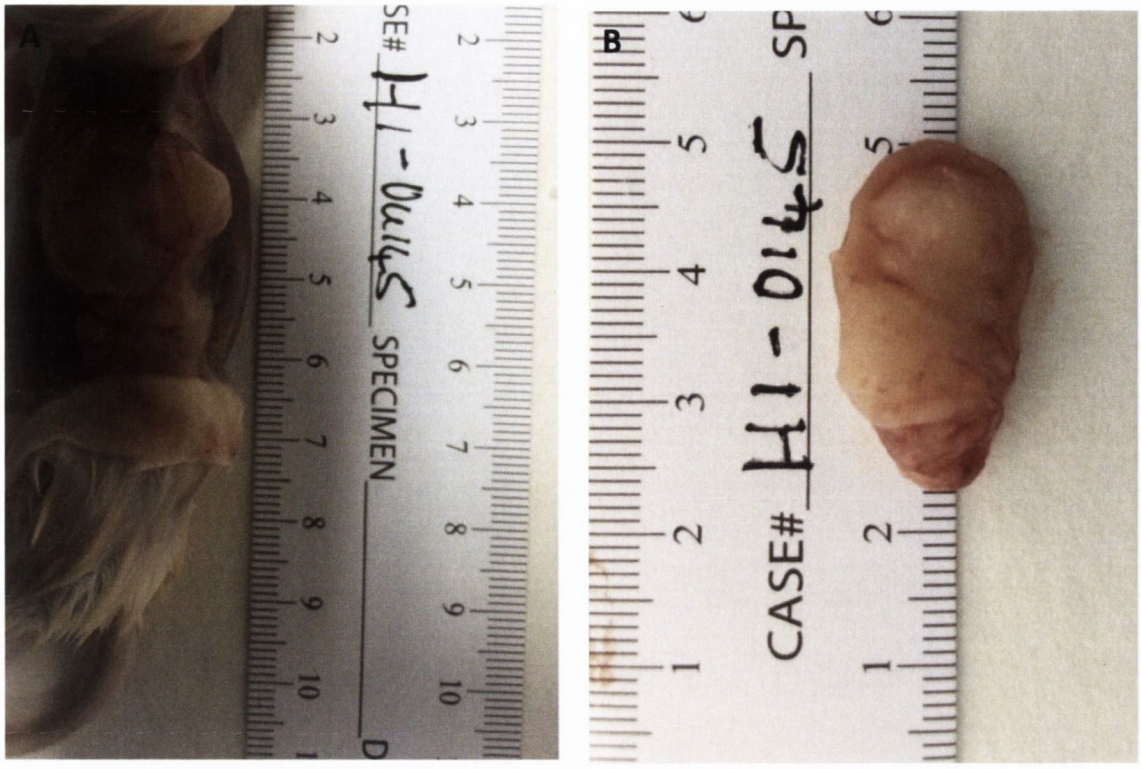

Figure 6.14 *DU145 holoclone-derived tumour.*

(A) DU145 holoclone-derived tumour protruding from flank of mouse prior to excision. (B) DU145 holoclone-derived tumour upon excision. Upon visual inspection holoclone-derived tumours were noted to be significantly larger than those originating from parental cells.

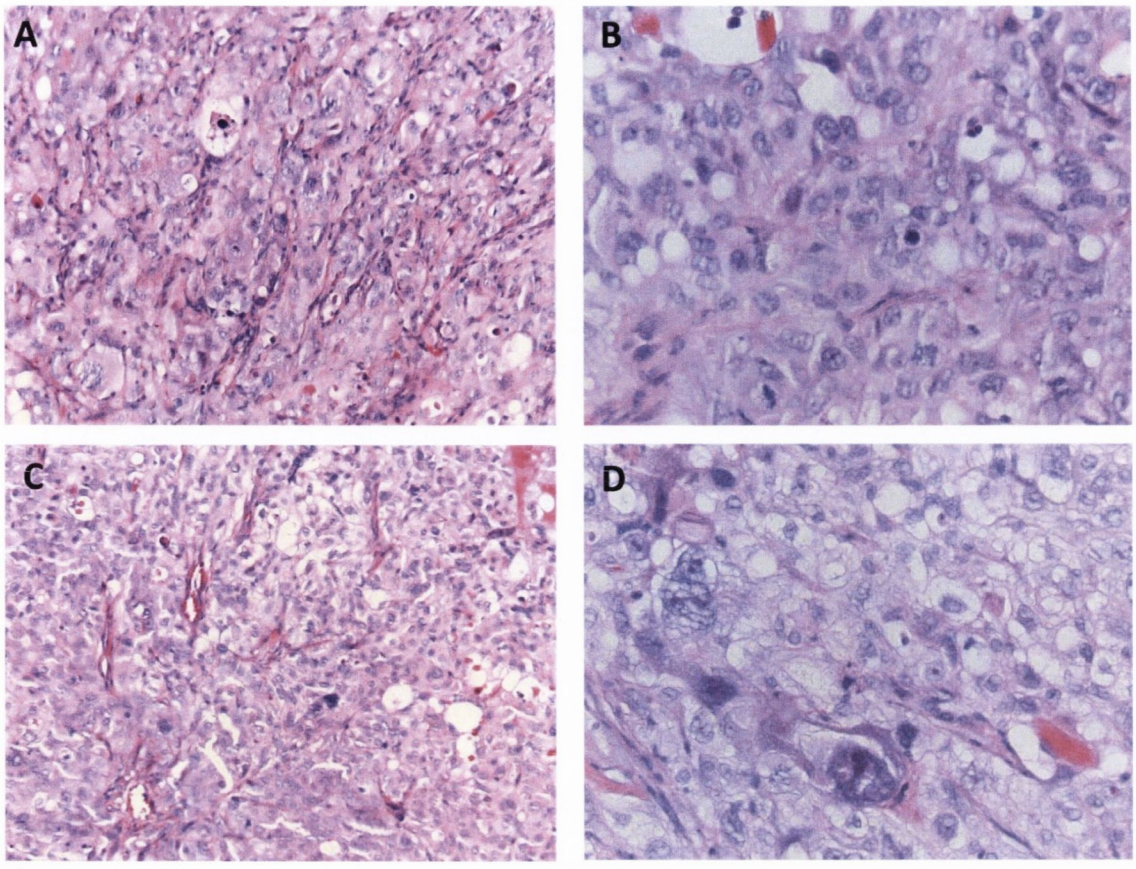

Figure 6.15 *Representative DU145 parent and holoclone derived H&E-stained tumour sections.*

(A) DU145 parent-derived tumour (20X). Muscle was found to be infiltrated by very poorly differentiated carcinoma with marked pleiomorphism. Tumour was comprised of mainly large cells with polylobated nuclei; some with prominent nucleoli, some multi-nucleated. Very apparent apoptosis and mitosis was noted. (B) DU145 parent-derived tumour (40X) (C) DU145 holoclone-derived tumour (20X). Tumours were histologically very similar to parental cell-derived tumours. However, focal areas of clear cell change were identified and muscle infiltration was found to be much more widespread. (D) DU145 holoclone-derived tumour (40X).

6.2.2.5 DU145 Holoclones Generate Significantly Larger Tumours

DU145 cell-derived tumour dimensions were sequentially measured by external calipers every four to five days following first palpation at 49 days. Tumour volume was calculated by use of the modified ellipsoid formula ½(Length x Width^2) (Euhus *et al.*, 1986). Tumours were harvested one week following the final *in vivo* tumour volume measurement and *ex vivo* dimensions were obtained by measurement with calipers. Tumour mass was also obtained *ex vivo* using a fine balance. DU145 holoclone-derived tumours were found to be of a larger volume at first palpation and this trend continued until excision (Figure 6.16). Upon excision, *ex vivo* holoclone tumour volumes were found to be statistically significantly larger than those generated by parental cells (Figure 6.17). Furthermore, DU145 holoclone-generated tumours were found to have a statistically significantly larger mass than parental-derived tumours (Figure 6.18).

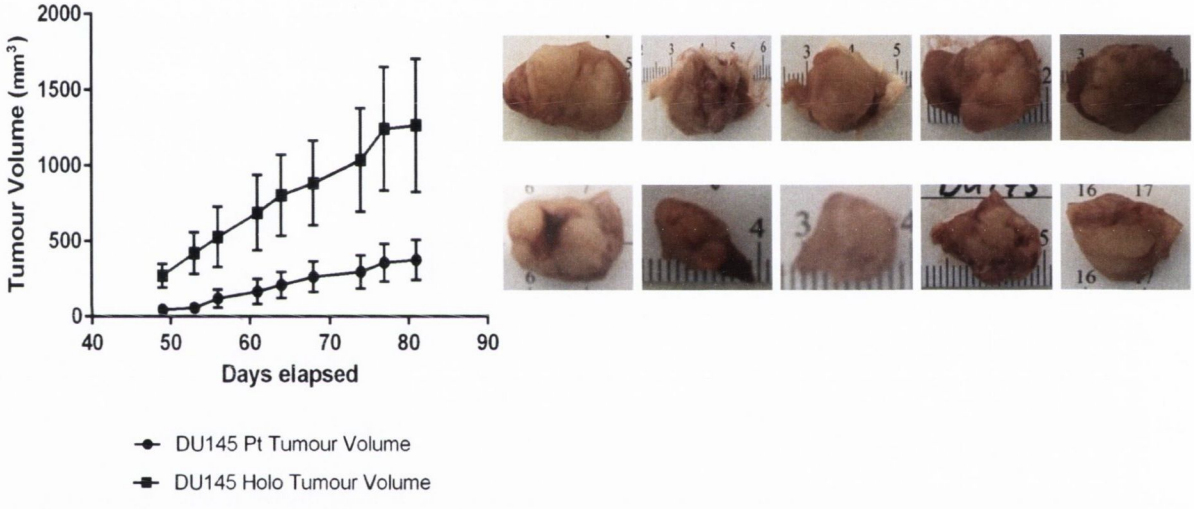

Figure 6.16 *Xenotransplantation growth curve following subcutaneous injection of 3000 DU145 parental and holoclone cells into NOD/SCID mice (n=6).*

Palpable tumours were noted in each group at 49 days following injection. Growth curves were stopped when one mouse from each group reached the predefined ethical limit of 20 mm x 20 mm palpable tumour. Representative tumours can be seen to the right of the growth curves. Error bars represent standard error of the mean.

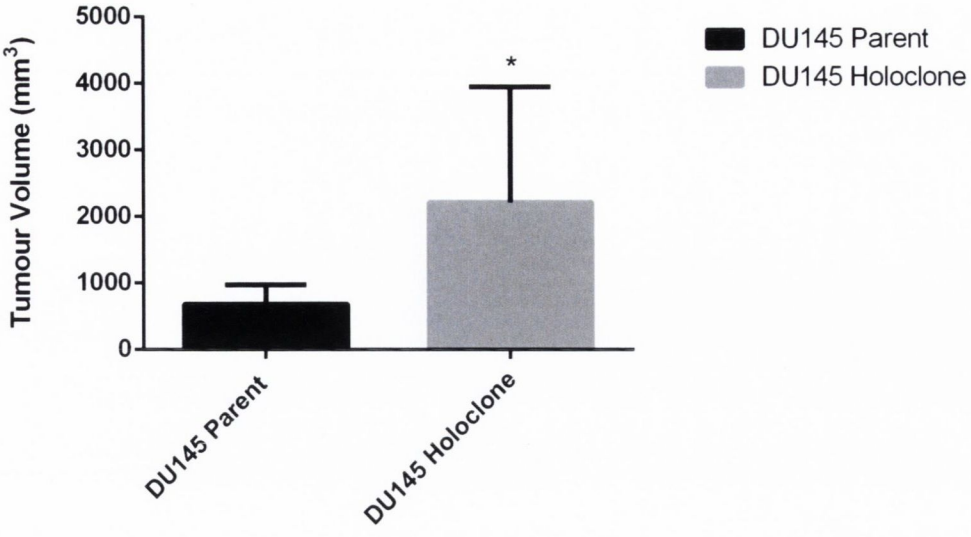

Figure 6.17 *DU145 parent and holoclone-derived tumour final volume upon excision 87 days following subcutaneous injection (n=6).*

Tumours derived from DU145 holoclones were found to be statistically significantly larger than those derived from parental cells. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

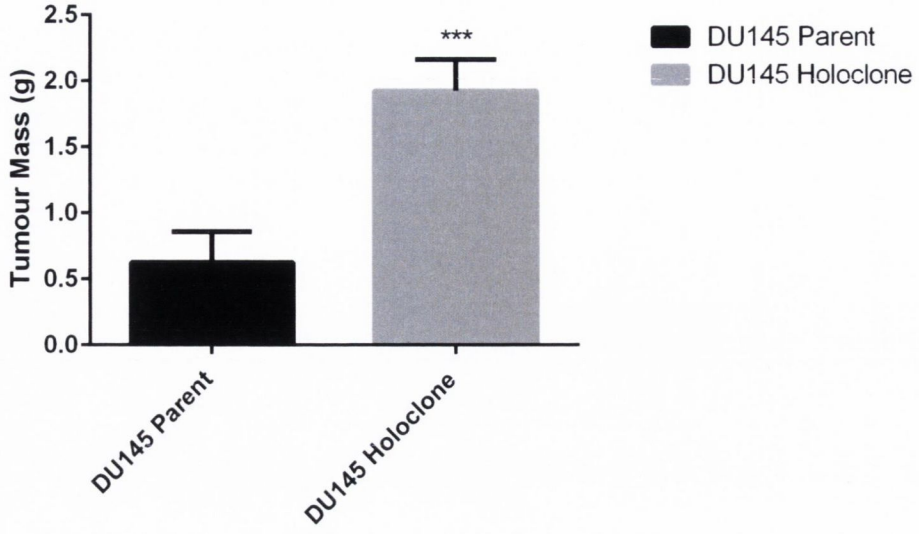

Figure 6.18 *DU145 parental and holoclone-derived tumour mass upon excision at 87 days following subcutaneous injection (n=6).*

A statistically significant difference in mass was observed between DU145 parental and holoclone tumours, with holoclone-derived tumours exhibiting a larger mass. Statistical significance: +/- 1.5-fold change, Student's two-tailed t test, $p < 0.05$. Data is graphed as mean and standard error of the mean.

6.2.2.6 Investigation of Stem Profile in DU145 Parent and Holoclone Xenograft Tumours

Stem-associated genes were found to be highly enriched in monoclonally-derived DU145 holoclones (Chapter 5). This stem profile was also found to persist within tumours derived from PC-3 holoclones (Figure 6.6). Thus, in order to determine whether the intrinsic stem phenotype of monoclonally-derived DU145 holoclones is maintained within daughter tumours, qRT-PCR was used to examined the expression of the same stem-associated gene panel; ALDH1, CD44, SOX2, POU5F1/OCT4, and NANOG. SOX2, POU5F1/OCT4 and NANOG were all found to be more highly expressed in DU145 holoclone-derived tumours, while both ALDH1 and CD44 were downregulated in holoclone-generated tumours (Figure 6.19). It is also noteworthy, that stem markers are more highly expressed in parental-derived tumours than was originally observed in parental cells (Chapter 5). Expression levels did not reach significance.

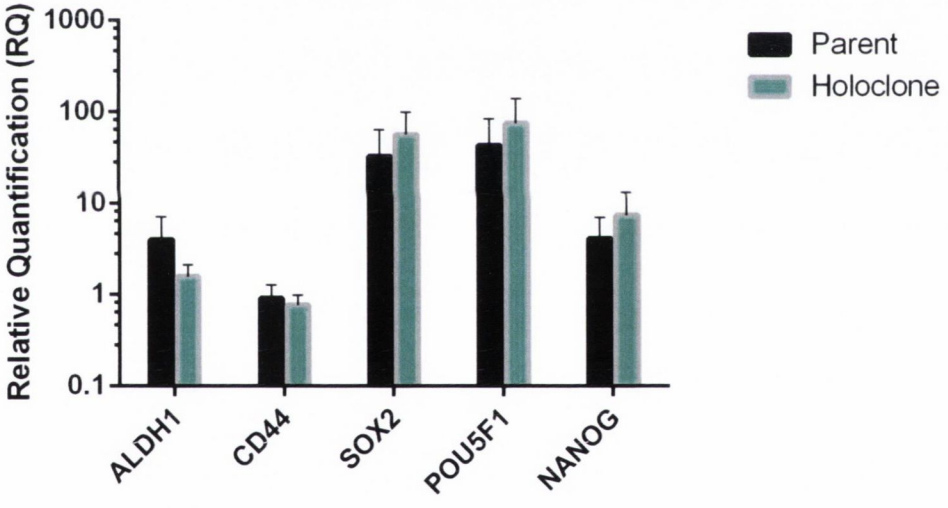

Figure 6.19 *Stemness gene expression analysis in DU145 parental and holoclone-derived xenograft tumours (n=4).*

Stem-associated markers SOX2, NANOG and POU5F1/OCT4 were found to be more highly expressed in tumours generated by DU145 holoclone cells than parental cells, while both ALDH1 and CD44 were downregulated in holoclone-derived tumours. Differential expression levels did not reach significance.

6.2.2.7 Investigation of EMT Marker Expression

In the absence of evident metastases upon dissection of the validation transplantation cohort, we sought to investigate whether holoclone-derived tumours preferentially exhibited a loss of epithelial morphology and a concomitant acquisition of a mesenchymal phenotype. Immunohistochemistry was employed to examine the expression of E-cadherin and vimentin in a subset of representative parent and holoclone-derived (n=3) tumour sections. The expression of E-cadherin was scored using a semi-quantitative intensity score from 0 to 3, where the most representative areas were chosen when there was any heterogeneity. A significant decrease in E-cadherin expression was observed in both PC-3 and DU145 holoclone tumours when compared to parental tumours. However, no detectable difference in vimentin expression was observed between parent and holoclone tumour pairs in either cell line.

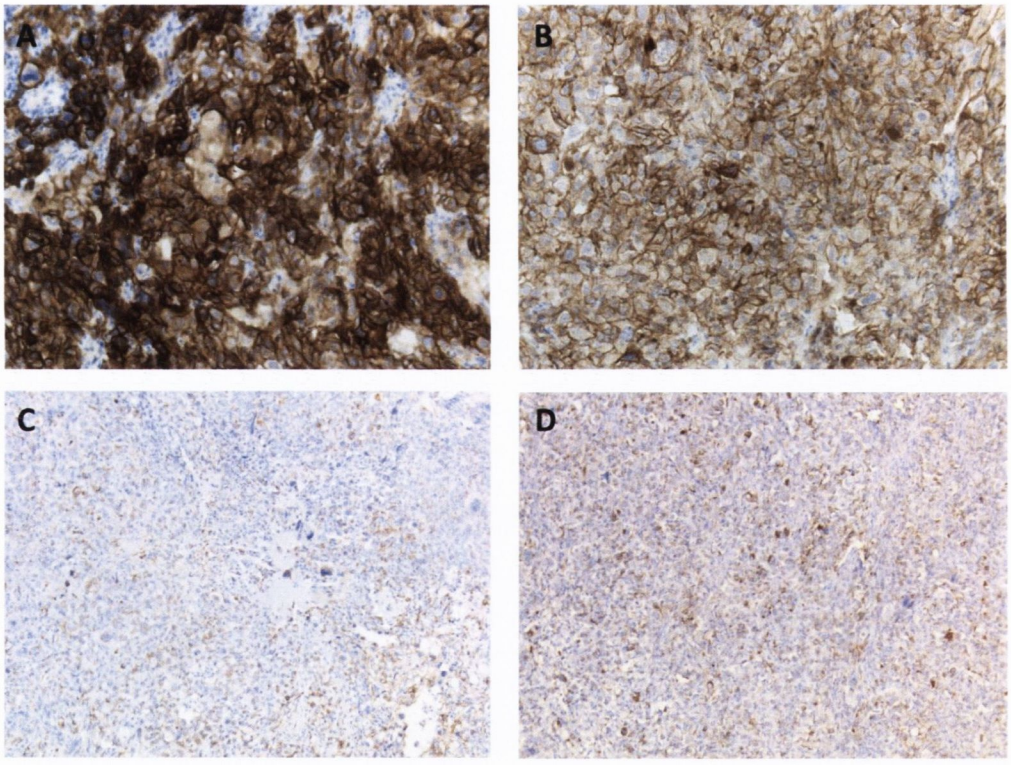

Figure 6.20 *Representative E-Cadherin and Vimentin staining in DU145 parent and holoclone-derived tumour sections.*

(A) E-cadherin expression in DU145 parental tumour (20X). (B) E-cadherin expression in DU145 holoclone-derived tumour. E-cadherin expression was found to be significantly diminished in the holoclone-derived tumour sections. (C) Vimentin expression in a DU145 parental tumour (10X). (D) Vimentin expression in a DU145 holoclone tumour (10X). Very little difference in vimentin expression was observed between parent and holoclone samples.

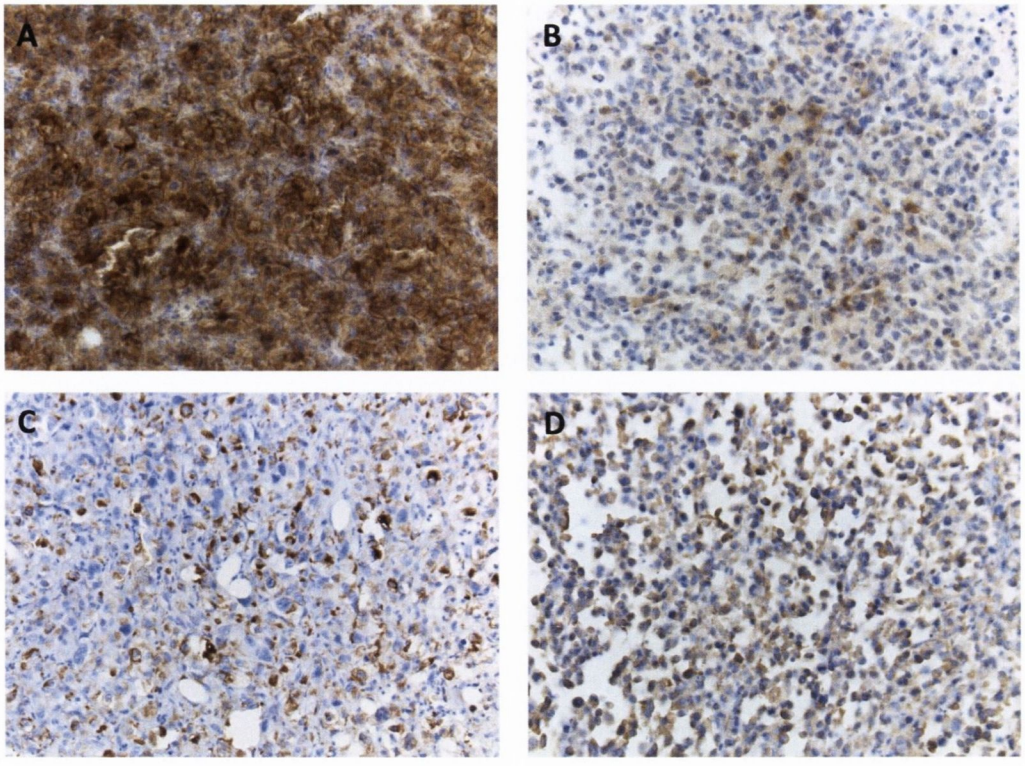

Figure 6.21 *Representative E-Cadherin and Vimentin staining in PC-3 parent and holoclone-derived tumour sections.*

(A) E-cadherin expression in PC-3 parental-derived tumour (20X). (B) E-cadherin expression in PC-3 holoclone-derived tumour (20X). E-cadherin expression was found to be markedly diminished between the tumour sections (C) Vimentin expression in PC-3 parental-derived tumour (20X). (D) Vimentin expression in PC-3 holoclone-derived tumour (20X). No markedly visible gain in vimentin expression was observed in the holoclone-generated tumour.

6.2.2.8 Investigation of CD34 Expression in Parent and Holoclone-derived Tumours

Cancer stem cells have previously been implicated in the development of angiogenesis (6.1.3). Thus, to determine whether a differential degree of vasculature was present between parental and holoclone-derived tumours, a subset of tumour sections (n=3) were chosen for immunohistochemical staining with the vascular marker CD34. However, no positive staining was observed in either PC-3 (Figure 6.22) or DU145 (not shown) tumour samples (parent and holoclone).

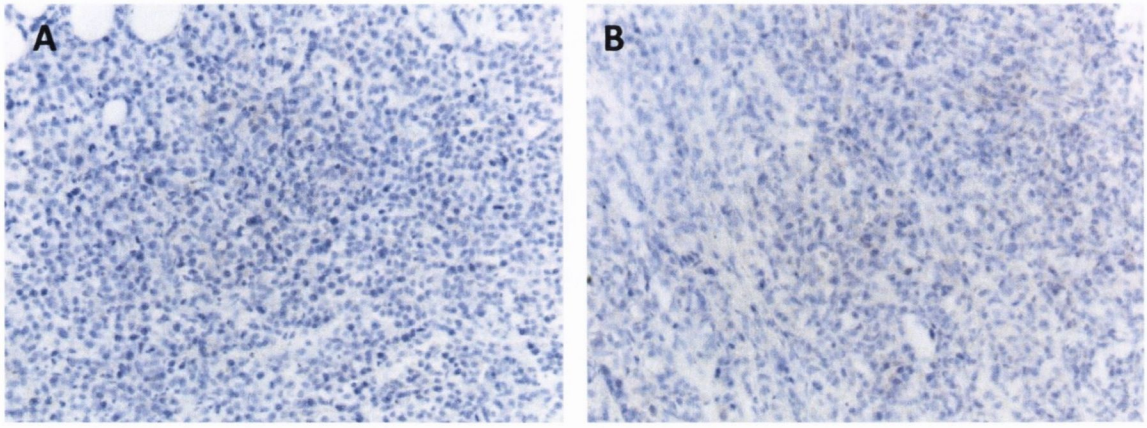

Figure 6.22 *Representative CD34 staining in PC-3 parent and holoclone-derived tumours.*

(A) CD34 staining in PC-3 parental cell tumour (20X). (B) CD34 staining in PC-3 holoclone-derived tumour (20X).

No detectable staining was observed in either parental or holoclone tumour samples (both PC-3 and DU145).

6.3 Discussion

The discovery that phenotypically plastic malignant cell subpopulations perpetuate tumour growth has spawned a new paradigm of oncogenesis. For many years, phenotypic and functional tumour heterogeneity has been implied by the disparate responses of patients to therapeutic regimens (Williams *et al.,* 2013). However, it was not until a number of technical advancements which facilitated the isolation of discrete cell populations in the 1990s, that the stochastic model of clonal evolution was redacted. Understanding this complex, interconnecting milieu of hierarchical highly proliferative, differentiating cells, influential stroma and infiltrating haematopoietic cells will surely expedite the accurate clarification of disease subtypes and may ultimately enable the development of effective personalised therapies. In recent years, technically feasible immunodeficient mouse strains which allow the engraftment of human tumour cells have become more widely available, and it has been postulated that *in vivo* xenograft models provide a more accurate reflection of human tumour behaviour than standard tissue culture techniques (Jin *et al.,* 2010). Indeed, xenograft transplantation has vested exceptional advancements in the understanding of tumorigenesis, however it has been postulated that the engraftment of established cell lines fails to accurately mimic the behaviour of naturally occurring tumours (Baiocchi *et al.,* 2010). The gold standard for confirmation of cancer stem cell identity is the ability to initiate *in vivo* tumours, which recapitulate the cellular heterogeneity of the primary tumour. This intrinsic ability to efficaciously regenerate original tumours *in vivo* has led to the suggestion that cancer stem cells represent improved preclinical models and an innovative approach for the development of pathway-targeted drugs. To this end, we sought to substantiate the cancer stem cell phenotype of our monoclonally-derived holoclones by performing xenotransplantation assays. Furthermore, we assessed the growth characteristics of derivative tumours to determine whether cancer stem cells play a preferential role in the development of metastasis and angiogenesis.

6.3.1 PC-3 and DU145 Holoclones Recapitulate the Histostructural Heterogeneity of Parental Tumours

Normal stem cells are responsible for the development and regeneration of tissue and organ systems (Weissman, 2000). These units of biological organisation, which fuel homeostasis are capable of both self-renewal and multilineage differentiation. Malignant stem cells are postulated to share the functional capabilities of normal stem cells, an idea which is supported by the observation that the mechanisms which regulate key features of normal tissue stem cells such as self-renewal, also frequently mediate tumorigenesis (Shackleton *et al.,* 2010). Thus, by analogy malignant cells which possess intrinsic self-renewal ability

should be capable of multilineage regeneration. We evaluated the tumorigenic potential of prostate cancer holoclones through the subcutaneous injection of PC-3 and DU145 holoclone cells mixed with matrigel membrane matrix in non-obese diabetic, severe combined immunodeficient (NOD/SCID) mice. The injection of as low as 1000 cells in preliminary assays and 3000 in validation studies consistently resulted in the growth of tumour xenografts with morphological features closely resembling the parental cell tumours, as demonstrated by haematoxylin and eosin staining of histologically prepared tumour sections (Figure 6.4, Figure 6.8, Figure 6.15). The gross similarity between differentiated parent cell- and holoclone-derived tumour xenografts demonstrates that prostate cancer holoclones can effectively reproduce the human disease in the mouse.

There is a significant degree of ambiguity surrounding the principles of cancer stem cell definition. The most stringent definition states that a cancer stem cell should be a single cell capable of reconstituting a tumour phenotypically similar to the parent in a recipient animal (Tang *et al.,* 2007). However, it is almost impossible to identify a single cell capable of fulfilling this criterion particularly when transplanted into a foreign host environment. Thus, in lieu of achieving this strict requirement, it has been hypothesised that in order to confirm cancer stem cell identity, the population in question must be prospectively purified, must be confirmed to possess the biologic properties of stem-like cells and *in vivo* tumorigenicity experiments should be performed to confirm enrichment of tumorigenic cells and reconstitution of histologically homogeneous tumours (Tang *et al.,* 2007). Our work to date has demonstrated that monoclonally-derived PC-3 and DU145 holoclones; are capable of self-renewal, possess a high proliferative capacity, preferentially express embryonic stem-associated genes, and finally can generate xenograft tumours which are phenotypically similar to those generated by differentiated parental cells.

6.3.2 PC-3 and DU145 Holoclones Preferentially Promote Metastasis

Epithelial mesenchymal transition (EMT) is the phenomenon whereby epithelial cells acquire a mesenchymal phenotype mediated by environmental cues. This highly conserved process is utilised by human malignant cells to invade surrounding tissues and is a crucial step in the metastatic cascade (Sampieri *et al.,* 2012). Seminal work by Thomas Brabletz provided a deeper understanding of the mechanisms involved in this process through the elegant demonstration of abundant nuclear β-catenin in colon cancer cells undergoing EMT along the invasive tumour front. These findings indicated that contact with stromal components can directly elicit EMT and facilitate the detachment and migration of tumour cells. Furthermore, it was demonstrated that single tumour cells located within the stromal compartment remained enriched for nuclear β-catenin and exhibited loss of E-cadherin and gain of

vimentin expression, two characteristics synonymous with a mesenchymal phenotype. These observations first engendered the concept of migrating cancer stem cells (Brabletz *et al.,* 2005). Indeed, the intrinsic plasticity of cancer stem cells is postulated to expedite their ability to undergo mesenchymal epithelial transition (MET), shedding the transient mesenchymal phenotype at distant sites and recrudescing to the original epithelial identity. Thus, it would appear that the modulation of E-cadherin expression is crucial to enabling cancer cell invasion. While a definitive link has been established between the presence of cancer stem cells within a primary lesion and the development of metastases, the putative mechanism of a causal relationship remains enigmatic. Herein, we provide preliminary data to indicate that prostate cancer holoclones play a preferential role in the development of metastasis in the mouse host. Initial feasibility xenotransplantation assays saw the development of perisplenic metastases in a NOD/SCID mouse subcutaneously injected with 1000 PC-3 holoclone cells. The epithelial identity of these metastatic lesions was confirmed by immunohistochemical staining with CAM 5.2, which reacts with human cytokeratins 8 and 18 (Figure 6.5). While the occurrence of metastasis was only observed in one mouse, these results provide *in vivo* evidence for the role of putative cancer stem cells in malignant dissemination. Dissection of the mice within our validation study provided no pathological evidence of distant metastatic spread in either the parent or holoclone groups; however a subset of parent and holoclone tumours were chosen for immunohistochemical analysis of E-cadherin and vimentin in order to assess the ability of holoclone cells to modulate E-cadherin expression *in vivo.*

While no differential vimentin expression pattern was observed between parent and holoclone derivative tumours, a significant loss of E-cadherin expression was observed in tumours derived from both PC-3 and DU145 holoclones (Figure 6.20, Figure 6.21). These findings indicate that holoclone cells are preferentially capable of modulating E-cadherin expression in order to facilitate an invasive phenotype. As previously mentioned, the cadherin switch has been well documented throughout the literature, whereby epithelial cells begin to progressively downregulate E-cadherin expression during EMT (Bae *et al.,* 2011). A study by Syed *et al.,* (2008) has demonstrated that DU145 E-cadherin-deficient cells display an enhanced migratory phenotype, providing further evidence reconciling the loss of E-cadherin expression with a concomitant increase in invasive capability. Thus, our results albeit preliminary, tentatively suggest that the ability to modulate E-cadherin expression is distinctly manifested in prostate cancer holoclone cells.

6.3.3 Putative Phenotypically and Functionally Divergent Cancer Stem Cell Subsets

Phenotypic and functional heterogeneity is a pervasive hallmark of mammalian cells *in vitro* and *in vivo* (Tang, D., 2012). Cellular heterogeneity is the definition given to the complex coexistence of adult stem cells, committed progenitors and cells at various stages of differentiation in the human organ. Parallels have long been drawn between organogenesis and tumorigenesis and in recent years the plasticity believed to define normal somatic stem cells has also been ascribed to cancer stem cells. This concept suggests that irrespective of the fractionation technique, the purified 'cancer stem cell population' contains a combination of true stem cells and proliferative mature progenitors (Tang, D., 2012). Evidence in support of this idea can be drawn from the most intensely studied cancer stem cell population; breast cancer stem cells (BCSC). BCSCs have been enriched using multiple strategies and cell surface markers; $CD44^+/CD24^{-/lo}$ (Al-Hajj *et al.,* 2003), side population (Engelman *et al.,* 2008), $ALDH1^-$ (Ginestier *et al.,* 2007), and PKH26 dye-retaining cells (Pece *et al.,* 2010). While the nature of the relationship between these varying tumorigenic subsets remains unclear, the observation that stem-like cells can be identified by distinct approaches indicates that a phenotypic plasticity exists between these populations. There is further evidence to suggest that breast cancer stem cell subsets differ from patient to patient and are infact determined by an individual's genetic background (Proia *et al.,* 2011). The disparate approach to the identification of cancer stem cells has also been observed in prostate cancer stem cell research, as tumour-initiating stem-like cells have been identified as $CD44^+$ (Patrawala *et al.,* 2006), $CD44^+/\alpha_2\beta_1$ (Patrawala *et al.,* 2007), $ALDH1^+$ (Li *et al.,* 2010) and holoclones (Li *et al.,* 2008). Thus, it is becoming increasingly understood that human tumours contain multiple, distinct pools of self-renewing cancer stem cells.

Monoclonal cultivation is postulated to derive the heterogeneity, which characterises normal epithelial stem cells in the form of holoclones, meroclones and paraclones. Holoclones are believed to represent the most highly proliferative, stem-like cells, however it could be postulated that behavioural plasticity present within the holoclone population may account for the disparity observed between PC-3 and DU145 holoclone-derived tumour growth characteristics and stem expression profiles. PC-3 and DU145 holoclones generated through monoclonal cultivation were found to highly express genes closely associated with the maintenance of stem cell characteristics including SOX2, POU5F1/OCT4 and NANOG (Chapter 5). In order to evaluate whether this stem expression profile was maintained in derivative tumours, qRT-PCR was employed to examine the expression of the same gene panel in formalin-fixed paraffin-embedded parent and holoclone-generated tumour sections. Expression patterns garnered from analysis of PC-3 feasibility study specimens indicated a faint reservation of stem characteristics in holoclone-derived tumours as evident by

increased expression of CD44, SOX2, POU5F1/OCT4 and NANOG when compared to parental cell-derived tumours (Figure 6.6). The distinct maintenance of a stem expression profile was also observed in DU145 holoclone-derived tumours (Figure 6.19). These findings suggested a subtle retention of intrinsic stem identity during clonal expansion of the population within the foreign host microenvironment. However, when this analysis was extended to a second round of PC-3 xenotransplantation assays, stem-associated genes were found to be more lowly expressed in PC-3 holoclone-derived tumours (Figure 6.12).

Furthermore, growth curves indicated that PC-3 holoclone-derived tumours were consistently smaller than parental tumours (Figure 6.9, Figure 6.10, Figure 6.11). These results are in stark contrast to DU145 transplantation growth curves, as DU145 holoclones were found to generate significantly larger, more invasive tumours than parental cells (Figure 6.16, Figure 6.17, Figure 6.18). This discrepancy in transplanted holoclone behaviour observed between a) studies of the same cell line and b) studies of two distinctive cell lines, may suggest a phenotypic and indeed functional divergence present within the cancer stem cell population. The observation that normal somatic stem cells are highly heterogeneous would appear to impute that the cancer stem cell population are also heterogeneous, particularly given the analogies which have long been drawn between normal stem cell and tumour development. The strategy of holoclone derivation employed by this study, monoclonal cultivation/colony forming assay, may indeed enrich for cells with a profoundly enhanced tumour-initiating capacity but this population of cells may be diluted by varying degrees of intrinsic plasticity due to the presence of a mixed population of true cancer stem cells and mature independently evolving tumorigenic clones (Odoux *et al.,* 2008). Conceivably, cancer stem cell plasticity may be caused by the experimental induction of differentiated cells into a stem-like state. For example, the culturing of malignant cells under low O_2 tension has been demonstrated to increase the expression of stem-associated genes and the percentage of cells which phenotypically resemble cancer stem cells (Koh *et al.,* 2011). As previously stated, the interrelationship existing between these disparate cell populations and indeed their relationship with differentiated tumour cells remains unclear and an appreciation of cancer stem cell plasticity will require a revision of previously accepted concepts. In order to further explore this idea, it will be necessary to investigate the behaviour of holoclones generated by multiple established prostate cancer cell lines to simultaneously compare the tumorigenic potential of different subsets. Both PC-3 and DU145 cells are inherently aggressive, metastatic AR^- lines and it could be postulated that cancer stem cell-containing holoclones generated by genotypically distinct cell lines behave in different ways. Furthermore, serial transplantation experiments should ideally be

performed in order to truly confirm the stem identity of these cells and to fully dissect functionally divergent oncogenic subgroups.

6.3.4 PC-3 and DU145 Holoclone-derived Tumours and Angiogenesis

Neovascularisation is critical for tumour growth and cancer stem cells are intimately involved in tumour progression. Thus, it has been hypothesised that cancer stem cells are promoters of tumour angiogenesis (Kaur *et al.,* 2005). As previously mentioned, a study by Bao *et al.,* (2006) has provided compelling evidence to support the role of cancer stem cells in angiogenesis. This study demonstrated that vascular endothelial growth factor (VEGF) is 10-20-fold more highly expressed in $CD133^+$ stem-like cells. Furthermore, the inhibition of VEGF with the human monoclonal antibody bevacizumab diminished the angiogenic advantage of this cellular fraction *in vitro.* These and other findings have indicated that cancer stem cells are strongly angiogenic, an activity which may be mediated by increased expression of VEGF. In order to investigate the relationship between putative prostate cancer stem cells and angiogenesis, we sought to ascertain whether holoclone-derived tumours were more highly vascularised than those originating from differentiated parental cells by staining with the vascular marker CD34. Our results in a subset of tumour samples indicated very little CD34-positive vascular area, thus it was impossible to determine whether holoclone-derived tumours demonstrated a significant increase in vascularity. Perhaps a qRT-PCR expression analysis of CD34 using mouse-specific primers would be a more appropriate method to determine a differential degree of vascularity between parental and holoclone-seeded tumours. Should PC-3 holoclone-derived tumours be demonstrated to exhibit a drastically increased vascularity compared to tumours derived from differentiated parental cells, this could at least in part, explain the growth disparity observed between parent and holoclone tumours. PC-3 holoclones, which have previously been implicated in the promotion of angiogenesis, may give rise to smaller xenograft tumours than parental cells as an increased angiogenic activity may cause the tumour to exhaust the available vascular supply at an accelerated rate (Zhang *et al.,* 2010).

6.4 Conclusion

From a clinical perspective, there is intense interest in targeting the cancer stem cell niche. However as the cancer stem cell concept is further explored, the more enigmatic this goal becomes. The disparity in cancer stem cell frequency among the same tumour types and the lack of concordance among markers, which fractionate this cellular population has defined a new paradigm of cancer stem cell identity (as discussed in Chapter 5). This population, which was once considered stable and distinct, is now postulated to represent a highly heterogeneous and perhaps functionally divergent subset (Tang, D., 2012). Moreover, it has

been suggested that a complex, reciprocal relationship exists between cancer stem cells and their differentiated progeny (Tang, D., 2012). As tumorigenesis progresses, the cancer stem cell phenotype may become altered in response to exogenous signals. Indeed, multiple anti-cancer therapeutics have been implicated in the enrichment of the cancer stem cell fraction by inducing dedifferentiation (Rich *et al.,* 2007; Dean *et al.,* 2005). Herein, we sought to further investigate the efficacy of our model of cancer stem cell propagation by exploring the *in vivo* tumorigenic behaviour of clonally-derived prostate cancer holoclones. A dissociated holoclone fraction and differentiated parental cells were injected into the flanks of NOD/SCID mice to determine the growth characteristics of a putatively 'pure' cancer stem cell population. We have demonstrated that PC-3 and DU145 holoclones generate 'histocopies' of parental cell tumours. In particular, DU145 holoclones generate much larger, more invasive tumours than differentiated parental cells indicating an altered behavioural pattern for prostate holoclones. However, the apparent divergence in *in vivo* growth characteristics observed between PC-3 and DU145 holoclones may suggest a phenotypic and functional heterogeneity present within the holoclone fraction. In order to address these issues and explore this concept further, it will be necessary to elucidate the relationship which exists between the cancer stem cell population as a whole and their differentiated counterpart. As previously suggested, the behaviour of cancer stem cells may be profoundly altered when they are removed from the bulk, differentiated population (Bissell *et al.,* 2005). However, a detailed genetic analysis will be paramount to elucidating the complex mechanisms which govern this 'moving target' cellular populace.

Next-Generation Sequencing of Prostate Cancer Holoclones and Derivative Tumour Xenografts

Chapter 7

Chapter 7. Next-generation Sequencing of Prostate Cancer Holoclones and Derivative Tumour Xenografts

7.1 Introduction

7.1.1 Non-coding RNAs

Approximately 90% of the human genome is actively transcribed into RNAs, however only 1.5 – 2% is postulated to comprise protein-coding genes creating a vast chasm of seemingly redundant genomic white noise. In recent years, this transcriptional desert within the human genome has adopted a rich functional spectrum with the identification of several crucial non-coding RNA (ncRNA) classes (Dunham *et al.,* 2012). The most widely investigated ncRNAs are miRNAs, whose oncogenic and tumour suppressive roles in human cancer have been well characterised. However, increasingly miRNAs are postulated to represent only a small fraction of the highly abundant newly identified ncRNA species, which include small interfering RNA (siRNA), PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), transcribed ultraconserved regions (t-UCRs) and large intergenic noncoding RNAs (lincRNAs).

ncRNAs can be loosely subclassified into two major categories based upon transcript size; small ncRNAs and long ncRNAs. Small ncRNAs which are generally less than 200 nucleotides in length, include the widely-documented miRNAs, siRNAs, piRNAs and the newly-identified transcription initiation RNAs (tiRNAs) (Gibb *et al.,* 2011). As previously discussed, miRNAs represent a crucial class of intricate gene expression modulators, whose pathogenic role has been well established in the cancer paradigm. In contrast to small ncRNAs, long non-coding RNAs are RNA transcripts ranging from 200 nucleotides to ~100 kilobases in length, a highly heterogeneous group of molecules which have been implicated in a broad gamut of biological processes (Huang *et al.,* 2013).

The fundamental aim of this chapter is to focus on the functional roles of genes, microRNAs and long ncRNAs in the context of phenotypically plastic stem-like cells. Dysregulation of ncRNAs has been implicated in the development of cancer and the pathologic activity of cancer stem cells subsets. Furthermore, lncRNAs in particular have been associated with the maintenance of self-renewal in embryonic and pluripotent stem cells. An improved understanding of the molecular mechanisms modulating ncRNA activity and how this RNA species influences both tumour phenotype and the maintenance of stem potential may facilitate the development of more effective prostate cancer therapies.

7.1.2 Non-coding RNAs in Cancer and Cancer Stem Cells

A recent report by Kapranov *et al.,* (2010) has estimated that the bulk RNA load within a cell, independent of ribosomal and mitochondrial RNA comprises 'dark matter', non-coding RNA transcripts whose function has yet to elucidated. However, the limited group of human long non-coding RNAs which have been characterised, have been implicated in a diversity of biological processes including epigenetic modification, alternative splicing, nuclear import and as modulators of mRNA decay (Orom *et al.,* 2010; Chen *et al.,* 2010). Furthermore, there is increasing reports of dysregulated lncRNA expression in a variety of cancer types, indicating that aberrant expression of this RNA species may play a critical role in the development and perpetuation of tumorigenesis (Huarte *et al.,* 2010). Indeed, the pique in interest surrounding ncRNAs in recent years has led to a surge in publications describing cancer associations, which has concomitantly spawned the development of a number of lncRNA databases. At present, the human genome is estimated to contain between 7,000 and 23,000 unique lncRNA genes indicating that this class of RNAs represents a vast, unappreciated, regulatory network (Lipovich *et al.,* 2010).

Among the better characterised lncRNAs, which have been implicated as regulators of oncogenic and tumour suppressive pathways, is lincRNA-HOTAIR (HOX antisense intergenic RNA). This 2.2kb gene is located in the mammalian homeobox C (HOXC) locus on chromosome 12q13.13 (Rinn *et al.*, 2007). HOTAIR was found to be highly upregulated in primary and metastatic breast tumours and this high level of expression was found to predict both metastasis and poor survival. Inhibition of this gene was found to alter the methylation of H3K27 and diminish the invasive capacity of cells, while restoration of HOTAIR had the opposite effect (Gupta *et al.,* 2010). Furthermore, the transplantation of cells expressing HOTAIR into mouse mammary fat pads was demonstrated to promote primary tumour growth (Gupta *et al.,* 2010). It has also been reported that multiple lncRNAs are transcribed from the HOX locus, indicating that HOTAIR may be part of a complex global regulatory infrastructure (Khalil *et al.,* 2009). HOTAIR functions in the repression of genes within the HOXD cluster by binding to the mammalian polycomb repressor complex (PRC)2 and recruiting it to the locus. This complex consists of the H3K27 methylase EZH2, SUZ12 and EED (Khalil *et al.,* 2009). Trimethylation of H3K27 results in the repression of thousands of genes involved in a diverse array of biological pathways including differentiation, maintenance of stem cell pluripotency, and as previously mentioned human cancer (Gupta *et al.,* 2010). While the precise mechanism of HOTAIR activity remains to be elucidated, there is much evidence to heavily implicate this RNA in the development of metastasis.

The MALAT1 (metastasis-associated lung adenocarcinomas transcript 1) gene was first identified during a screen of both primary and metastatic non-small cell lung cancer lesions (Diederichs *et al.,* 2003). Overexpression of this gene located at chromosome 11q13.1, has since been associated with high metastatic potential and poor patient outcome in non-small cell lung cancer (NSCLC). Furthermore, elevated expression levels of this RNA have been identified in multiple human cancer types, including breast, prostate, colon, liver and uterus (Guffanti *et al.,* 2009; Yamada *et al.,* 2006; Lin *et al.,* 2007; Luo *et al.,* 2006). Interestingly, the MALAT1 locus is situated within an area on chromosome 11q13.1 known to harbour chromosomal translocation breakpoints associated with cancer (Davis *et al.,* 2003). A number of studies have suggested a role for MALAT1 in the regulation of cell mobility. A study by Tano *et al.,* (2010) has demonstrated that RNA interference (RNAi)-mediated knockdown of MALAT1 diminishes the *in vitro* migration capacity of lung adenocarcinomas cells. This observed abrogation in cellular motility has been postulated to occur as a result of the negative regulation of mobility-associated genes through both transcriptional and post-transcriptional mechanisms. These findings have been replicated using cervical cancer cells lines (Guo *et al.,* 2010). Similar to HOTAIR, there is an abundance of evidence to suggest that MALAT1 expression is associated with the invasive potential of metastatic cancer cells.

Many of the lncRNAs which have been well characterised are known to be expressed in multiple cancer types, however only a select few have been exclusively associated with a single cancer type. A recent study by Yang *et al.,* (2013) has reported the overexpression of two lncRNAs in prostate cancer; PRNCR1 (prostate cancer non-coding RNA 1) and PCGEM1 (prostate specific gene 1). These RNAs are situated in a 'gene desert' on chromosome 8q24.2 (Gibb *et al.,* 2011). PRNCR1, a 13kb transcript, was found to bind to the caroboxy-terminally acetylated androgen receptor resulting in the recruitment of the DOT1L enzyme and subsequent sequestration of PCGEM1 to the amino terminus of the androgen receptor. PCGEM1 was found to induce PYGO2, a critical component of the Wnt signalling complex. This interaction activates the expression of AR-targeted genes through binding to H3K4me3 chromatin marks in the promoter sequences of target genes. Thus, binding of these RNAs strongly enhances ligand-dependent and ligand-independent androgen receptor-mediated gene activation programs. Furthermore, short hairpin RNA-targeting of these lncRNAs in androgen-refractory prostate cancer cell lines was found to strongly inhibit *in vivo* tumour-forming potential. These findings indicate that the overexpression of PRNCR1 and PCGEM1 may be associated with the development of castration resistance in prostate carcinoma.

Maternally expressed gene 3 (MEG3) was the first tumour-suppressive lncRNA to be identified. This gene is expressed in many normal tissues with the highest expression levels

observed in the brain and pituitary gland (Zhang *et al.,* 2003). The undetectable expression of MEG3 in human cancer cell lines has led to the postulation that this gene functions in the suppression of cell growth. Indeed, forced overexpression of MEG3 results in the inhibition of cancer cell growth, further substantiating its tumour suppressive role (Zhang *et al.,* 2010). MEG3 is a maternally imprinted gene comprising 10 exons, located on human chromosome 14q32.3 (Miyoshi *et al.,* 2000). Functionally, MEG3 has been implicated in the activation of p53 and facilitation of p53 signalling, including enhancing the interaction between p53 and the promoter regions of it transcriptional targets (Zhou *et al.,* 2007). Hypermethylation of the MEG3 promoter region has been observed in a variety of cancer types including pituitary tumours and is proposed as the mechanism mediating loss of MEG3 expression (Zhang *et al.,* 2003). In contrast, lincRNA-p21 has been identified as a downstream repressor in the p53 pathway (Huarte *et al.,* 2010). In murine lung cancer, linc-p21 is induced by p53 and functions to repress the expression of p53 target genes, through the association with hnRNP-K, a protein which binds the promoter sequences of these genes (Huarte *et al.,* 2010). While lincRNA-p21 appears to be highly conserved in humans, it remains to be determined whether this gene acts via an analogous mechanism.

7.1.3 Long ncRNAs in Cancer Therapeutics

Similar to the therapeutic potential of miRNAs, long non-coding RNAs represent a currently unexploited stratum of bioregulation, in terms of novel biomarkers and the development of strategic targeted therapies. Many lncRNAs have been identified as differentially expressed in a tissue- and cancer-specific manner, which supports their prospective utility as diagnostic and prognostic biomarkers. For example, the prostate cancer-specific lncRNA DD3 has demonstrated exceptional functionality as a fluid-based marker of disease. Furthermore, this amplification-based marker has demonstrated higher specificity than the current diagnostic indicator in clinical practise, serum prostate specific antigen (PSA) (Hessels *et al.,* 2003). In comparison to their smaller counterpart, exploration into the utilisation of lncRNAs as therapeutic agents is in its infancy and advancements within this field are heavily reliant upon gaining a deeper understanding of the functional mechanisms of this RNA species. However, a number of unique characteristics of lncRNAs make them optimal candidates for therapeutic intervention. Many lncRNAs appear to be functionally restricted by secondary structure, which provides a potential route for inhibitions. It has been hypothesised that by focusing on the cancer-specific transcript (e.g. HOTAIR) to target the RNA-protein interface could provide a viable method of lncRNA inhibition. Abolishing the interaction between HOTAIR and the PRC2 complex by the administration of an "antagolinc" could in theory normalise the chromatin state and diminish the metastatic capacity of tumorigenic breast cells (Tsai *et al.,* 2011) (Figure 7.1).

A plasmid construct, harbouring a diphtheria toxin gene driven by H19-specific regulatory sequences has demonstrated promise in reducing bladder tumour size in early human trials (Amit *et al.,* 2010). The H19 lncRNA is strongly expressed in embryonic cells and its levels have been demonstrated as highly abundant in a variety of human cancers (Poirier *et al.,* 1991; Gibb *et al.,* 2011). Administration of this plasmid is postulated to enhance DNA uptake via clathrin-dependent and –independent pathways, resulting in the expression of diphtheria toxin in tumour cells, which ultimately reduces bulk tumour size. These significant advancements in the utilisation of lncRNA-targeting provide a glimpse at the potential of this RNA class as novel strategies in both cancer diagnostics and therapeutics.

The widespread dysregulation of ncRNAs has been experimentally demonstrated in multiple human cancer types. However, at present there remains a vast swathe of RNA species whose contribution to tumour phenotype remains obscure. While long ncRNAs have been implicated in the maintenance of self-renewal in embryonic and pluripotent stem cells, and their expression is known to be dysregulated in various solid tumours, it is not yet known what role they play in the tumorigenic process or if their dysregulated expression disturbs the cancer stem cell population. Thus, a deeper understanding of the biology of ncRNAs is crucial to the absolute exploitation of non-coding transcripts as therapeutic tools.

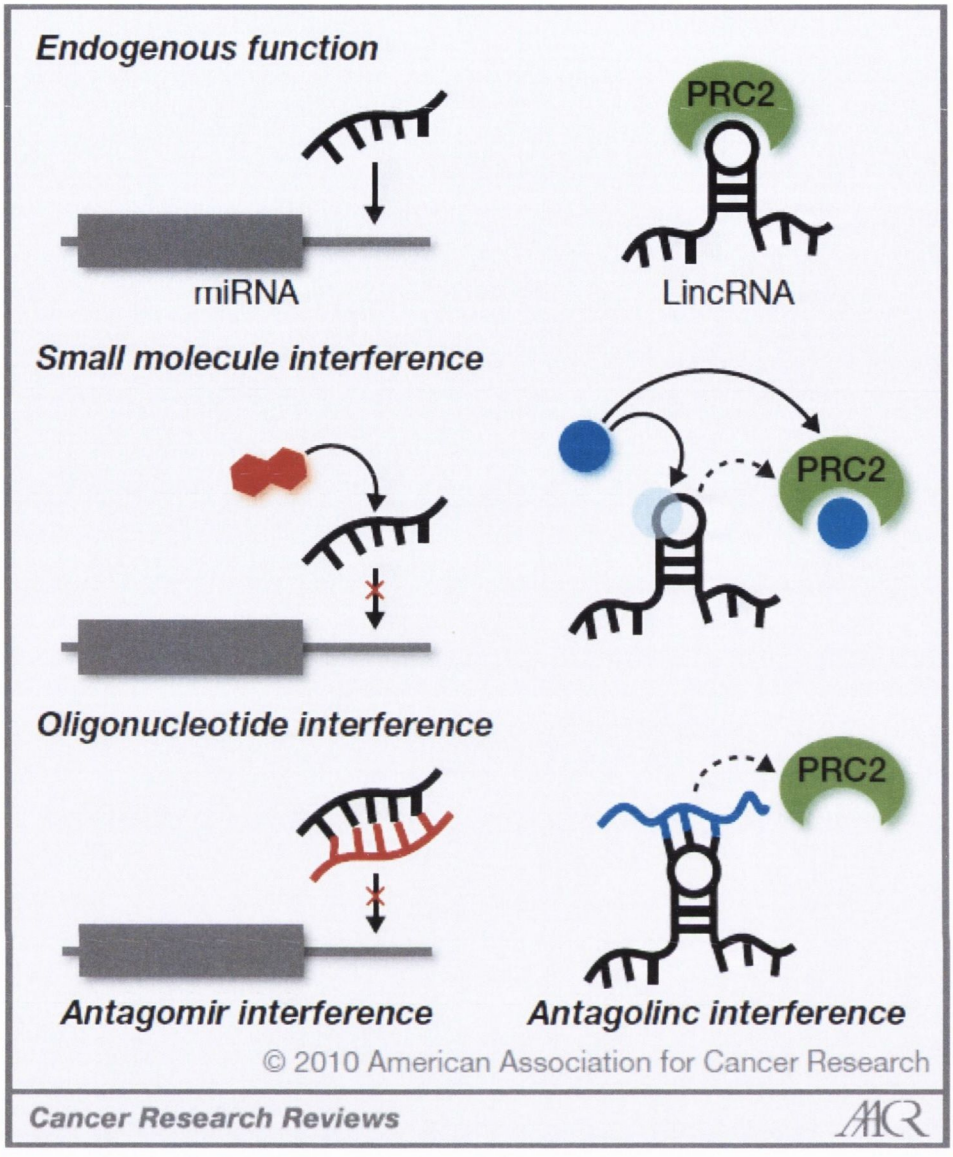

Figure 7.1 *Targeting of cancer-specific ncRNAs.*

Small molecules have been identified which bind to miRNAs, concomitantly disrupting base pairing. It has been hypothesised that targeting lincRNAs, which act as structural scaffolds, such as HOTAIR, could inhibit binding to chromatin modifying complexes without disrupting the binding of different RNAs. Adapted from Tsai *et al.,* (2011).

7.1.4 Experimental Hypothesis and Aims

In recent years, genome-wide association studies, particularly the ENCODE project, have stimulated a dramatic reinterpretation of the functional significance of the human genome (Dunham *et al.,* 2012). Rather than enclaves of protein-coding genes amidst a sea of junk DNA, it is now widely accepted that a great deal of the human genome encodes critical regulatory information (Cheetham *et al.,* 2013). While only ~1.2% of the genome is protein-coding, the remaining 90% represents a vast transcriptional landscape which serves as a template for innumerable regulatory non-coding RNAs. These dynamic modulators of biological function exhibit exquisite cell-specific expression patterns (Dinger *et al.,* 2008). The discovery of such a rich functional spectrum embodied within the human genome has refined the cancer paradigm, with many hypothesising that non-coding regulatory sequences account for a significant proportion of the genetic etiology of malignancy. A number of studies have indicated that cancer risk loci are located outside of protein-coding regions, in noncoding RNA transcripts suggesting that noncoding loci also play a critical role in tumorigenesis. To this end, the hypothesis of this work was that unique ncRNA signatures act as modifiers of prostate cancer stem cell properties. Thus, we sought to perform an exhaustive genetic analysis of the coding and non-coding transcripts of monoclonally-derived holoclones and their derivative xenograft tumours.

The fundamental objectives of this work were;

a) to gain a deeper understanding of the regulatory mechanisms, which underpin functional plasticity within the stem cell population and b) to identify the myriad of molecular alterations within coding and non-coding regions of the genome, which characterise this cellular population.

Therefore the primary aims of this study were;

- To perform 75bp paired-end next-generation sequencing of the long non-coding RNA and mRNA repertoires of PC-3/DU145 parent and monoclonally-derived holoclone cells and their respective xenograft tumours.

- To perform 50bp single-end next-generation sequencing of the small non-coding RNA repertoires of PC-3/DU145 parent and monoclonally-derived holoclone cells and their respective xenograft tumours.

7.2 Results

7.2.1 Differential Gene Expression Profiles

Global gene expression was analysed among cellular parent and holoclone samples (PC-3 and DU145) and their respective derivative murine tumours on the Illumina® HiSeq 2500 system. In total 8 samples were sequenced on two paired-end HiSeq 2500 lanes. Following the sequencing run the data was assessed and analysed by the in-house bioinformatician. The data passed all primary analyses and any sequencing errors were identified and filtered from the final dataset. Reads were mapped to the reference genome (Ensembl, build GRCh38, release 76) and sequences were annotated based on their overlap with publicly available mRNA transcripts. The number of reads that mapped to loci of known transcripts were used to calculate abundances, and therefore infer the expression levels of those transcripts within a given sample. These loci and other transcript information were provided by Ensembl for the longer RNAs, while miRBase provided the annotation for the small RNA analysis.

Read counts across the transcriptome were subsequently used to determine differential expression patterns between samples. Read counts were calculated across all transcripts using HTSeq. Transcripts which received low average counts (≤100) across samples were excluded in order to promote evidence-based results. Differential expression analysis was performed using edgeR and $\log_2$ fold change was calculated internally by edgeR analysis. It should be noted that, as with microarrays and other expression tests, biological replicates are required to perform statistical differential expression analysis (and indeed any statistical analysis) between phenotypes. In this experiment, the lack of biological replicates means the results are restricted to a descriptive analysis of the two cell lines and various conditions under study. As a result, individual differential expression test results were limited in their utility, and additional filtering of results was necessary. To identify the most biologically relevant differences between parent and holoclones, the results from PC-3 and DU145 analysis were intersected, with the overlapping genes postulated to represent the most pertinent molecular alterations (Figure 7.2, Figure 7.3). Forty-one genes were identified as upregulated in holoclones compared to parental cells (of both cell lines), while 12 genes were identified as downregulated in holoclones (Table 7.1, Table 7.2). In murine samples, two genes were found to be upregulated in holoclone tumours compared to parental cell-derived tumours. In addition, five genes were found to be downregulated in holoclone-derived tumours (Table 7.3, Table 7.4). Full (non-intersected) lists of differentially expressed genes can be found in Appendix 2. Notably, genes highly upregulated in holoclones include

EGR1, KITLG, NR4A2 and TXNIP, all of which have well-documented associations with haematopoiesis.

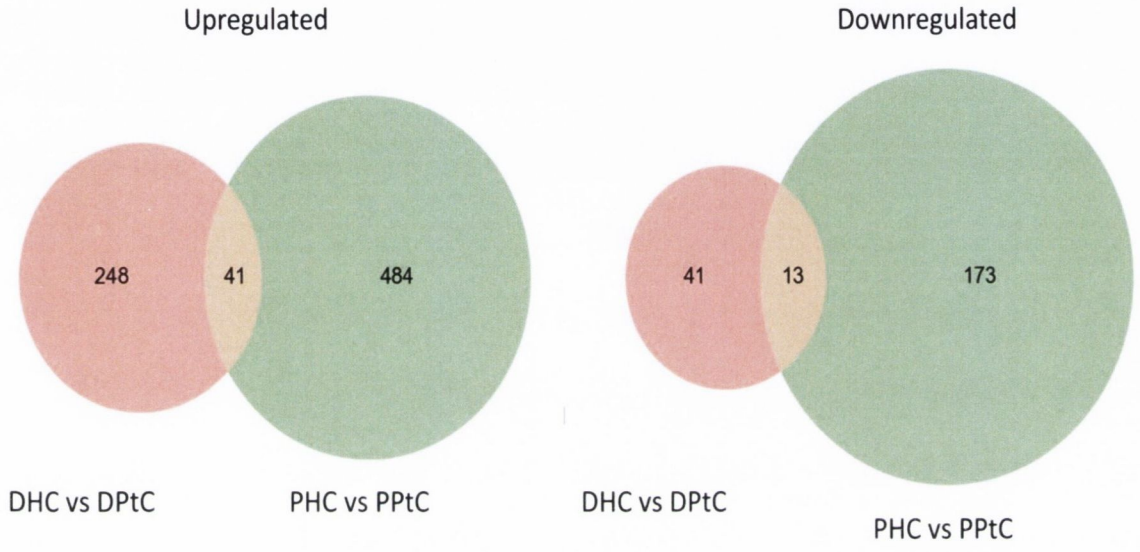

Figure 7.2 *Venn diagrams detailing shared and distinct gene expression among DU145 and PC-3 parent and holoclone cellular samples.*

41 genes were found to be commonly upregulated in holoclones (when compared to their parental counterpart) derived from DU145 and PC-3 cells. While 13 downregulated genes were shared by holoclones of PC-3 and DU145 cells. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells.

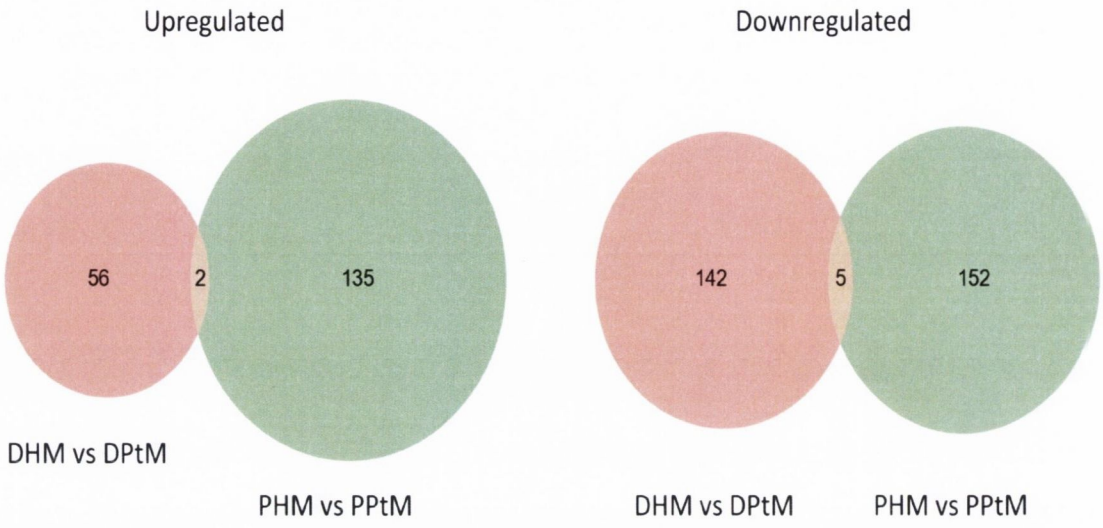

Figure 7.3 *Venn diagrams detailing shared and distinct gene expression among DU145 and PC-3 parent and holoclone murine tumour samples.*

Two genes were found to be commonly upregulated in PC-3 and DU145 holoclone-derived tumours (when compared to those generated by respective parental cells). While 5 genes were commonly downregulated in PC-3 and DU145 holoclone-derived tumours. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour.

DPtC (RC)	DHC (RC)	log_2FC	PPtC (RC)	PHC (RC)	log_2FC	Gene Symbol	Gene Name
98	414	1.861870187	136	477	1.896209439	KAL1	Kallmann syndrome 1 sequence
680	2644	1.743270355	333	1422	2.180718075	KITLG	KIT ligand
1251	7606	2.388275166	44	174	2.067126503	CYBRD1	cytochrome b reductase 1
26	152	2.326684791	749	2517	1.83531452	OAS1	2'-5'-oligoadenylate synthetase 1
195	2723	3.587193498	141	1729	3.701780532	CEMIP	cell migration inducing protein
1600	12140	2.707863208	193	698	1.940740011	MEGF9	multiple EGF-like-domains 9
303	3095	3.136388593	242	900	1.98117031	SCNN1A	sodium channel, non voltage gated 1 alpha subunit
125	681	2.229003734	296	1933	2.793461033	MAN1A1	mannosidase, alpha, class 1A, member 1
463	1825	1.762885088	691	3086	2.245593748	BCL6	B-cell CLL/lymphoma 6
63	347	2.243750732	59	284	2.35140018	PLCH1	phospholipase C, eta 1
1125	6444	2.302240165	7	214	4.995458319	EGR1	early growth response 1
3288	24578	2.686369381	942	4268	2.266430436	CLU	clusterin
34	1195	4.914921178	26	340	3.789163949	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
156	601	1.729398943	141	477	1.844169319	LYPD3	LY6/PLAUR domain containing 3
101	1261	3.424970326	111	1331	3.669163266	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
366	2677	2.654645015	155	677	2.212773422	CCDC64	coiled-coil domain containing 64
15	130	2.89021127	66	208	1.740899874	KCNMB4	potassium channel subfamily M regulatory beta subunit 4

113	768	2.5478867	616	2395	2.045620329	SLC22A23	solute carrier family 22, member 23
264	1234	2.008584534	226	745	1.807156123	ARRB1	arrestin, beta 1
58	325	2.268363585	116	428	1.969119528	ARRDC4	arrestin domain containing 4
7	654	6.30676962	853	3511	2.127921838	SELENBP1	selenium binding protein 1
232	1203	2.158208803	104	328	1.742685818	HHIPL2	HHIP-like 2
263	1147	1.908592169	485	4288	3.23073151	NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
25	133	2.190549621	124	618	2.402877846	SLC2A12	solute carrier family 2
253	1773	2.592760012	167	779	2.307713253	ZNF117	zinc finger protein 117
163	694	1.873646386	34	177	2.462502739	NR4A2	nuclear receptor subfamily 4, group A, member 2
9148	34002	1.678405726	2001	6267	1.733814333	LPCAT1	lysophosphatidylcholine acyltransferase 1
40	194	2.05914422	282	907	1.771767071	B3GNT7	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7
287	1087	1.705153145	93	578	2.720898827	GBP2	guanylate binding protein 2, interferon-inducible
266	1960	2.665154064	493	1884	2.02068093	TP53INP1	tumor protein p53 inducible nuclear protein 1
150	1062	2.607146638	142	721	2.429868745	C10orf10	chromosome 10 open reading frame 10
51	230	1.954965468	68	943	3.877932813	PCSK9	proprotein convertase subtilisin/kexin type 9
902	9626	3.199899146	148	3711	4.73375688	FOS	FBJ murine osteosarcoma viral oncogene homolog
17	1113	5.807492711	83	415	2.406938577	GPX2	glutathione peroxidase 2
34	168	2.085434598	75	794	3.48874751	MX2	MX dynamin-like GTPase 2

88	611	2.578345873	62	316	2.433970228	SNN	stannin
9	150	3.826087649	145	1632	3.578168306	IFITM1	interferon induced transmembrane protein 1
202	4825	4.361642925	29	133	2.279049996	AKR1C1	aldo-keto reductase family 1, member C1
1883	7569	1.791333618	1015	4008	2.068088845	SLC5A3	solute carrier family 5 (sodium/myo-inositol cotransporter), member 3
44	175	1.773425575	10	141	3.887231057	PCDHGB2	protocadherin gamma subfamily B, 2
16	926	5.629011952	376	5955	4.071657779	TXNIP	thioredoxin interacting protein

Table 7.1 *Shared upregulated genes in PC-3 and DU145 holoclones.*

Log_2FC: Log_2 Fold Change. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells. RC refers to read count.

DPtC (RC)	DHC (RC)	log_2FC	PPtC (RC)	PHC (RC)	log_2FC	Gene Symbol	Gene Name
16203	5624	-1.742241365	16474	3967	-1.967212347	ASNS	asparagine synthetase
1078	359	-1.801589589	522	126	-1.962768943	IL11	interleukin 11
8561	2569	-1.952189565	47969	8653	-2.383988286	STC2	stanniocalcin 2
96	25	-2.150785786	98	26	-1.822627236	VGF	VGF nerve growth factor inducible
196	55	-2.046355204	633	175	-1.767319431	WNT10A	wingless-type MMTV integration site family, member 10A
559	118	-2.458391633	5668	152	-5.132753596	IL24	interleukin 24
1853	434	-2.309412605	2435	276	-3.053798858	HOXB9	homeobox B9
4548	1080	-2.289731278	6080	931	-2.620235511	DDIT3	DNA-damage-inducible transcript 3
623	205	-1.818604152	962	270	-1.745797468	CST6	cystatin E/M
80	24	-1.946671057	146	31	-2.144443285	GALNT9	polypeptide N-acetylgalactosaminyltransferase 9
8998	3033	-1.784486591	9987	1898	-2.308672397	UPP1	uridine phosphorylase 1

Table 7.2 *Shared downregulated genes in PC-3 and DU145 holoclones.*

Log_2FC: Log_2 Fold Change. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells. RC refers to read count.

DPtM (RC)	DHM (RC)	log_2FC	PPtM (RC)	PHM (RC)	log_2FC	Gene Symbol	Gene Name
101	859	3.004787878	260	1076	2.122406375	BMP2	bone morphogenetic protein 2
67	313	2.139912199	11	222	4.392918669	RNF150	ring finger protein 150

Table 7.3 *Shared upregulated genes in PC-3 and DU145 holoclone-derived murine tumours.*

Log_2FC: Log_2 Fold Change. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour. RC refers to read count.

DPtM (RC)	DHM (RC)	log_2FC	PPtM (RC)	PHM (RC)	log_2FC	Gene Symbol	Gene Name
513	146	-1.894059348	2675	517	-2.297172295	S100P	S100 calcium binding protein P
1494	153	-3.368479316	5747	1138	-2.262326566	MUC6	mucin 6, oligomeric mucus/gel-forming

Table 7.4 *Shared downregulated genes in PC-3 and DU145 holoclone-derived murine tumours.*

Log_2FC: Log_2 Fold Change. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour. RC refers to read count.

7.2.2 Integrative Analysis of Gene Networks Over-represented in Holoclones

In order to identify enriched biological themes within this dataset, the computationally-derived lists of genes dysregulated in holoclones (as outlined in 7.2.1) were examined by Gene Ontology (GO) classifications using DAVID (Database for Annotation, Visualisation and Integrated Discovery) (Table 7.5, Table 7.6, Table 7.7).

As gene ontology terms are naturally interconnected, it is logical to represent them as a hierarchical framework (Figure 7.4, Figure 7.5, Figure 7.6). A close relationship exists between many gene ontology terms, while some are subcategories of others. Thus, in order to represent the full breadth of biological function represented by the GO terms, which arose during this analysis, they were converted to a set of 'slim' terms prior to mapping. These 'GO slims' are a pre-compiled, condensed interpretation of the gene ontologies. 'GO slims' offer a broad, more granular overview of the molecular terminology without the fine details of individual gene ontology terms.

Gene ontology terms over-represented by PC-3 and DU145 holoclones include; organ development, haematopoiesis, regulation of apoptosis, regulation of cell communication, response to stress and anatomical structure development.

GO Term	Gene Name	Fold Enrichment
response to stimulus	EGR1, IFITM1, CLU, PCSK9,FOSB, TNFSF10, STC2, CYBRD1,SCNN1A, SNN, NR4A2, GPX2,DDIT3, NFKBIZ, AKR1C1, BCL6,KAL1, GBP2, KCNMB4, VGF,ARRB1, OAS1, TXNIP, TP53INP1,ASNS, MX2, FOS, IL11	2.4536
response to chemical stimulus	KAL1, EGR1, CLU, PCSK9,KCNMB4, VGF, STC2, CYBRD1,TXNIP, ASNS, NR4A2, GPX2,DDIT3, AKR1C1, FOS	3.5933
response to external stimulus	KAL1, ARRB1, TXNIP, ASNS,CLU, NR4A2, PCSK9, DDIT3,NFKBIZ, FOS, STC2, IL11	4.0289
response to inorganic substance	TXNIP, NR4A2, KCNMB4, DDIT3,FOS, CYBRD1	8.9815
response to stress	CLU, PCSK9, VGF, TXNIP, SNN,TP53INP1, ASNS, GPX2, NR4A2,MX2, DDIT3, NFKBIZ, FOS,BCL6, IL11	2.7318
response to extracellular stimulus	ASNS, NR4A2, PCSK9, DDIT3,FOS, STC2	8.3692
cellular response to extracellular stimulus	ASNS, NR4A2, PCSK9, FOS	19.1793
response to endogenous stimulus	TXNIP, ASNS, NR4A2, PCSK9,DDIT3, VGF, FOS	5.3039
response to oxidative stress	TXNIP, CLU, GPX2, DDIT3, FOS	9.3558
cell communication	ASNS, NR4A2, PCSK9, KCNMB4,TNFSF10, VGF, FOS, STC2, IL11	3.474
regulation of apoptosis	TXNIP, TP53INP1, ASNS, CLU,NR4A2, PCSK9, DDIT3, TNFSF10,BCL6	3.4351
regulation of programmed cell death	TXNIP, TP53INP1, ASNS, CLU,NR4A2, PCSK9, DDIT3, TNFSF10,BCL6	3.4013
regulation of cell death	TXNIP, TP53INP1, ASNS, CLU,NR4A2, PCSK9, DDIT3, TNFSF10,BCL6	3.3887
positive regulation of cellular process	HOXB9, EGR1, CLU, PCSK9,TNFSF10, TXNIP, KITLG,TP53INP1, ASNS, NR4A2, DDIT3,FOS, BCL6, IL11	2.326
response to protein stimulus	EGR1, NR4A2, DDIT3, FOS	11.4718
response to amine stimulus	ASNS, NR4A2, DDIT3	23.6054
response to organic substance	TXNIP, EGR1, ASNS, NR4A2,PCSK9, DDIT3, VGF, FOS	3.4049
positive regulation of biological process	HOXB9, EGR1, CLU, PCSK9,TNFSF10, TXNIP, KITLG,TP53INP1, ASNS, NR4A2, DDIT3,FOS, BCL6, IL11	2.1132
system development	KAL1, CST6, HOXB9, EGR1, CLU,PCSK9, VGF, SLC5A3, TXNIP,KITLG, NR4A2, DDIT3, FOS,BCL6, IL11	1.9756
positive regulation of apoptosis	TXNIP, TP53INP1, PCSK9, DDIT3,TNFSF10, BCL6	4.2819
positive regulation of programmed cell death	TXNIP, TP53INP1, PCSK9, DDIT3,TNFSF10, BCL6	4.2522
positive regulation of cell death	TXNIP, TP53INP1, PCSK9, DDIT3,TNFSF10, BCL6	4.2327
multicellular organismal development	KAL1, CST6, HOXB9, EGR1, CLU,PCSK9, FOSB, VGF, SLC5A3,TXNIP, KITLG, NR4A2, DDIT3,WNT10A, FOS, BCL6, IL11	1.8209
positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	HOXB9, KITLG, EGR1, NR4A2,DDIT3, FOS, IL11	3.4424
cellular response to stimulus	ARRB1, ASNS, NR4A2, PCSK9,DDIT3, AKR1C1, FOS, BCL6	2.9938
multicellular organismal process	KAL1, CST6, HOXB9, EGR1, CLU,KCNMB4, PCSK9, FOSB, VGF,SLC5A3, ARRB1, SCNN1A,TXNIP, KITLG, NR4A2, GPX2,DDIT3, WNT10A, AKR1C1, FOS,BCL6, IL11	1.5774
regulation of cell communication	ARRB1, KITLG, EGR1, PCSK9,KCNMB4, TNFSF10, VGF, BCL6,IL11	2.6607
positive regulation of nitrogen compound metabolic process	HOXB9, KITLG, EGR1, NR4A2,DDIT3, FOS, IL11	3.3355
response to toxin	NR4A2, AKR1C1, FOS	15.0919
positive regulation of macromolecule biosynthetic process	HOXB9, KITLG, EGR1, NR4A2,DDIT3, FOS, IL11	3.2845
response to organic nitrogen	ASNS, NR4A2, DDIT3	14.6128
positive regulation of macromolecule metabolic process	HOXB9, KITLG, EGR1, NR4A2,PCSK9, DDIT3, FOS, IL11	2.8646
positive regulation of cellular metabolic process	HOXB9, KITLG, EGR1, NR4A2,PCSK9, DDIT3, FOS, IL11	2.7897
positive regulation of cellular biosynthetic process	HOXB9, KITLG, EGR1, NR4A2,DDIT3, FOS, IL11	3.1359
anatomical structure development	KAL1, CST6, HOXB9, EGR1, CLU,PCSK9, VGF, SLC5A3, TXNIP,KITLG, NR4A2, DDIT3, FOS,BCL6, IL11	1.8215
response to insecticide	NR4A2, AKR1C1	87.677
positive regulation of biosynthetic process	HOXB9, KITLG, EGR1, NR4A2,DDIT3, FOS, IL11	3.0908
response to reactive oxygen species	TXNIP, DDIT3, FOS	12.2748
response to nutrient levels	ASNS, PCSK9, DDIT3, STC2	6.2309
positive regulation of metabolic process	HOXB9, KITLG, EGR1, NR4A2,PCSK9, DDIT3, FOS, IL11	2.6655
regulation of transcription from RNA polymerase II promoter	HOXB9, EGR1, NR4A2, FOSB,FOS, BCL6, IL11	2.9547
regulation of multicellular organismal process	KITLG, EGR1, NR4A2, PCSK9,KCNMB4, VGF, SLC5A3, BCL6	2.62
response to abiotic stimulus	ARRB1, TXNIP, SNN, VGF, FOS	4.1694
developmental process	KAL1, CST6, HOXB9, EGR1, CLU,PCSK9, FOSB, VGF, SLC5A3,TXNIP, KITLG, NR4A2, DDIT3,WNT10A, FOS, BCL6, IL11	1.6572
positive regulation of transcription from RNA polymerase II promoter	HOXB9, EGR1, NR4A2, FOS, IL11	4.1357
homeostatic process	GPX2, PCSK9, KCNMB4, DDIT3,VGF, AKR1C1, BCL6	2.8603
positive regulation of transcription	HOXB9, EGR1, NR4A2, DDIT3,FOS, IL11	3.2646
response to biotic stimulus	IFITM1, GPX2, MX2, DDIT3, FOS	3.9957
positive regulation of gene expression	HOXB9, EGR1, NR4A2, DDIT3,FOS, IL11	3.169
immune system process	OAS1, KITLG, GBP2, EGR1, CLU,TNFSF10, BCL6, IL11	2.4599
hemopoiesis	KITLG, EGR1, BCL6, IL11	5.2012
regulation of respiratory gaseous exchange	NR4A2, SLC5A3	47.2107
lymphocyte differentiation	EGR1, BCL6, IL11	8.9379
organ development	HOXB9, CST6, TXNIP, KITLG,EGR1, CLU, PCSK9, DDIT3, VGF,BCL6, IL11	1.9422
hemopoietic or lymphoid organ development	KITLG, EGR1, BCL6, IL11	4.7211

Table 7.5 *Table of full GO terms derived from gene ontology analysis using DAVID (biological processes).*

GO Term	Gene Name	Fold Enrichment
extracellular space	KAL1, KITLG, CLU, PCSK9, TNFSF10,VGF, IL24, IL11	3.7916
extracellular region part	KAL1, KITLG, CLU, PCSK9, WNT10A,TNFSF10, VGF, IL24, IL11	3.0436
extracellular region	KAL1, CST6, KITLG, CLU, PCSK9,WNT10A, TNFSF10, VGF, STC2, HHIPL2, IL24, IL11	1.9382
cell fraction	ARRB1, SCNN1A, OAS1, ASNS, MAN1A1,PLCH1, TNFSF10, FOS	2.3982

Table 7.6 *Table of full GO terms derived from gene ontology analysis using DAVID (cellular component).*

Gene targets in green represent those identified as upregulated while gene targets in red were found to be downregulated.

GO Term	Gene Name	Fold Enrichment
sequence-specific DNA binding	HOXB9, EGR1, NR4A2, DDIT3, FOSB,FOS, BCL6	3.8807
transcription factor activity	HOXB9, ZNF117, EGR1, NR4A2,DDIT3, FOSB, FOS, BCL6	2.7611
receptor binding	KITLG, PCSK9, TNFSF10, VGF, STC2,IL24, IL11	2.6587
enzyme inhibitor activity	KAL1, ARRB1, CST6, TXNIP	4.9853
calcium ion binding	GALNT9, LPCAT1, PCDHGB2,MAN1A1, PCSK9, MEGF9, PLCH1	2.5632

Table 7.7 *Table of full GO terms derived from gene ontology analysis using DAVID (molecular function).*

Gene targets in green represent those identified as upregulated while gene targets in red were found to be downregulated.

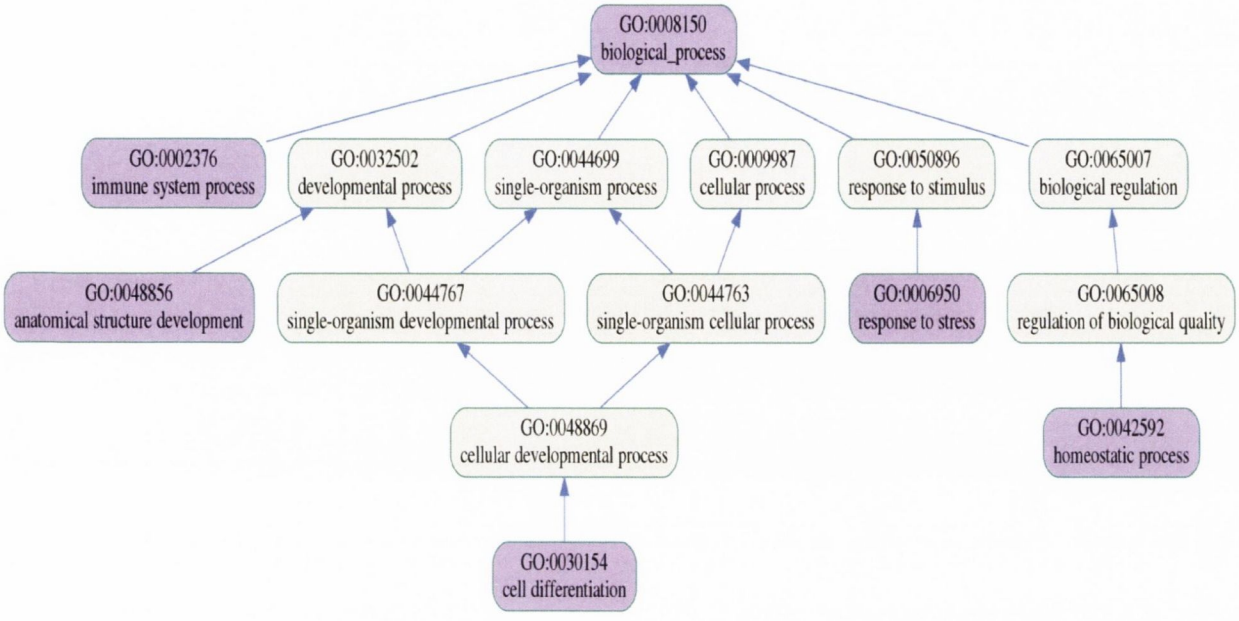

Figure 7.4 *Lineage map of gene ontology terms over-represented in PC-3 and DU145 holoclones (biological processes).*

This hierarchical network represents the biological processes over-represented in PC-3 and DU145 holoclones.

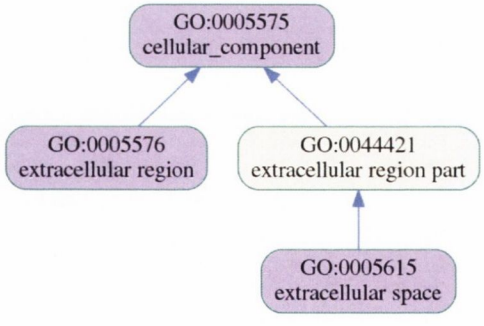

Figure 7.5 *Lineage map of gene ontology terms over-represented by PC-3 and DU145 holoclones (cellular component).*

This hierarchical framework represents the cellular component ontology, which describes gene product locations at the levels of subcellular structures and macromolecular complexes.

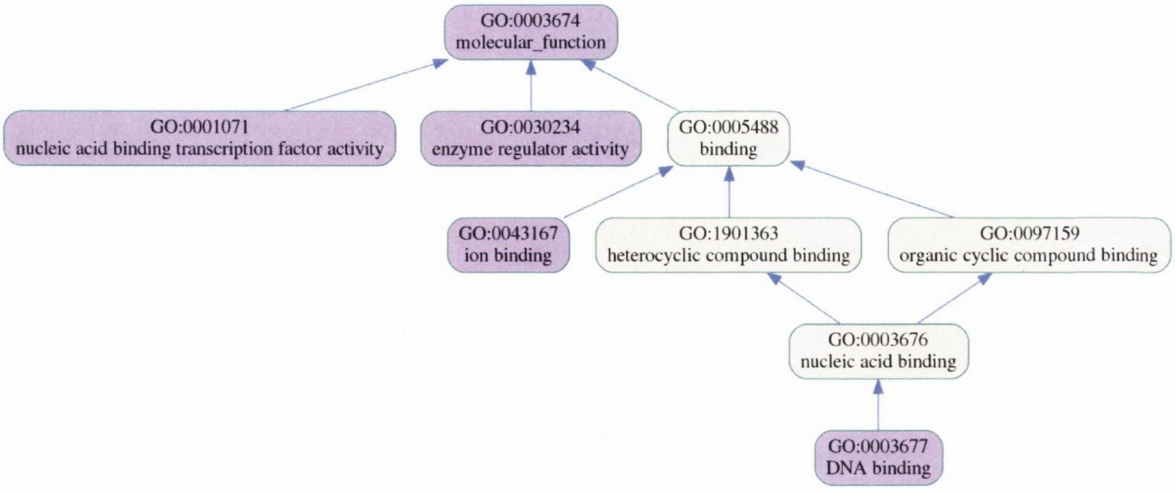

Figure 7.6 *Lineage map of gene ontology terms over-represented by PC-3 and DU145 holoclones (molecular function).*

This hierarchical network describes the precise molecular functions of gene products over-represented in PC-3 and DU145 holoclones. This differs from the biological processes a gene product is involved in, which include more than one activity.

7.2.3 Differential miRNA Expression Profiles

Similar to the analysis of mRNA data, the number of reads mapping to loci of known small RNA transcripts were utilised to quantify microRNA expression. These loci were provided by miRBase and read counts across the transcriptome were subsequently used to determine differential expression patterns between samples. Read counts were calculated across all transcripts using HTSeq and transcripts which received low average counts (≤100) across samples were excluded. Differential miRNA expression analysis was performed using edgeR. Results were filtered as described in 7.2.1, in order to identify the most biologically relevant changes in miRNA expression between parent and holoclone samples (Figure 7.7, Figure 7.8) (Table 7.8, Table 7.9, Table 7.10, Table 7.11).

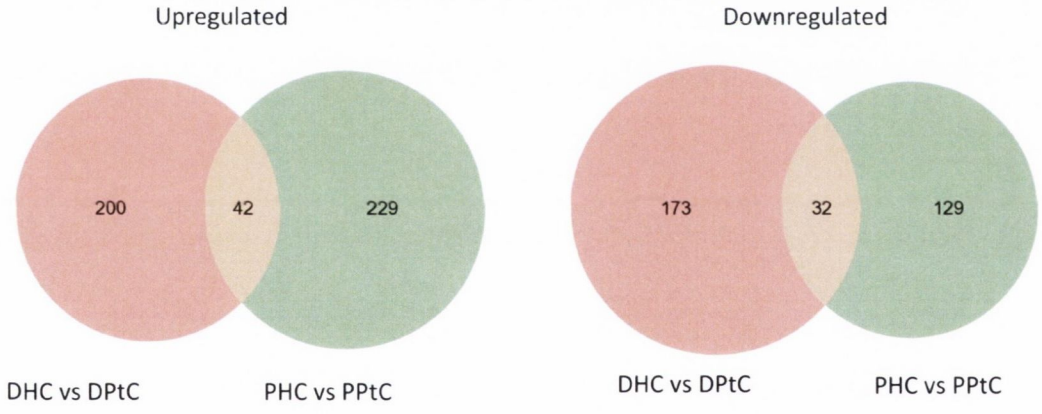

Figure 7.7 *Venn diagrams detailing shared and distinct miRNA expression among DU145 and PC-3 parent and holoclone cellular samples.*

Forty-two miRNAs were identified as upregulated in holoclones of both cell lines, while 32 miRNAs were commonly downregulated. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells.

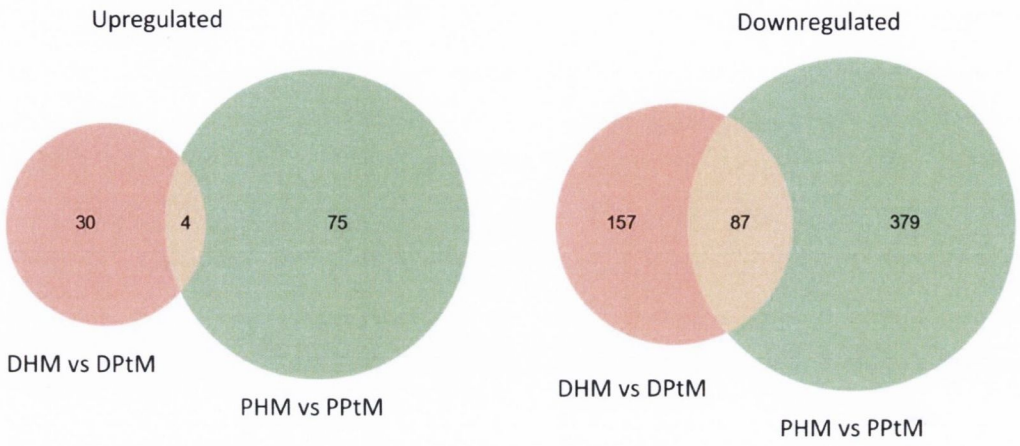

Figure 7.8 *Venn diagrams detailing shared and distinct gene expression among DU145 and PC-3 parent and holoclone murine tumour samples.*

Only 4 miRNAs were found to be commonly upregulated by holoclone-derived tumours, while a much larger set of 87 miRNAs were found to be downregulated in holoclone-derived tumours. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour.

DPtC (RC)	DHC (RC)	log_2FC	PPtC (RC)	PHC (RC)	log_2FC	miRNA
22	150	2.931697	0	101	9.409691	hsa-miR-6765-5p
6	281	5.688422	5	588	6.294994	hsa-miR-619-5p
13	291	4.640237	4	315	5.709631	hsa-miR-4632-5p
3	438	7.297761	2	140	5.505362	hsa-miR-6127
20	137	2.937659	3	165	5.180574	hsa-miR-6893-5p
13	201	4.106675	4	215	5.158933	hsa-miR-4783-3p
6	245	5.490723	2	107	5.118018	hsa-miR-664a-3p
37	118	1.839308	6	189	4.400082	hsa-miR-4766-5p
8	281	5.28119	15	384	4.114739	hsa-miR-2682-3p
22	151	2.941275	16	398	4.073877	hsa-miR-5703
13	164	3.813365	6	144	4.008119	hsa-miR-6759-5p
18	285	4.144751	5	111	3.891305	hsa-miR-6793-3p
2	132	6.123068	6	130	3.860722	hsa-miR-6750-5p
169	608	2.01603	26	480	3.647106	hsa-miR-7155-3p
7	158	4.640309	14	207	3.322586	hsa-miR-3928-3p
25	520	4.541052	29	417	3.287225	hsa-miR-500a-3p
60	210	1.974816	25	300	3.025662	hsa-miR-6800-3p
36	144	2.165716	85	975	2.964187	hsa-miR-7111-3p
5	180	5.303008	15	171	2.948325	hsa-miR-573
13	174	3.898697	11	125	2.940663	hsa-miR-6865-3p
0	205	10.76772	20	224	2.924914	hsa-miR-4706
74	363	2.462094	38	400	2.838442	hsa-miR-4279
204	2131	3.553865	95	974	2.802423	hsa-miR-146b-5p
26	912	5.295143	44	451	2.800528	hsa-let-7f-1-3p

26	337	3.859175	85	853	2.771362	hsa-miR-876-3p
70	318	2.35124	68	658	2.718477	hsa-miR-6090
16	118	3.042028	36	347	2.711249	hsa-miR-6720-3p
19	325	4.256708	37	308	2.499903	hsa-miR-665
81	370	2.359467	66	543	2.484414	hsa-miR-6794-5p
25	308	3.785696	38	310	2.470866	hsa-miR-3940-5p
257	818	1.839629	190	1519	2.44424	hsa-miR-6785-5p
28	204	3.028931	67	518	2.394771	hsa-miR-6746-3p
38	156	2.203378	13	92	2.260073	hsa-miR-1252-3p
53	166	1.814375	40	275	2.224309	hsa-miR-3944-3p
43	673	4.133848	3026	20449	2.202326	hsa-miR-375
43	166	2.115195	55	345	2.092878	hsa-miR-494-5p
28	473	4.241729	15	91	2.039369	hsa-miR-5096
50	354	2.990247	42	244	1.981641	hsa-miR-744-3p
31	434	3.971431	63	338	1.867734	hsa-miR-653-3p
129	570	2.312241	34	173	1.789931	hsa-miR-6752-3p
112	494	2.309484	242	1167	1.715123	hsa-miR-340-3p
224	730	1.87361	15239	71570	1.677398	hsa-miR-10b-5p

Table 7.8 *Shared upregulated miRNAs in PC-3 and DU145 holoclones.*

Log_2FC: Log_2 Fold Change. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells. RC refers to read count.

DPtC (RC)	DHC (RC)	log_2FC	PPtC (RC)	PHC (RC)	log_2FC	miRNA
457	2	-7.58417	356	0	-11.7797	hsa-miR-20b-5p
2937	521	-2.3249	4186	11	-9.10677	hsa-miR-27a-5p
214	8	-4.55146	1433	5	-8.67489	hsa-miR-454-5p
657	33	-4.14068	639	4	-7.82142	hsa-miR-26a-2-3p
191	1	-7.24813	143	1	-7.51505	hsa-miR-934
149	5	-4.69514	252	2	-7.42857	hsa-miR-4269
129	21	-2.4425	432	5	-6.9452	hsa-miR-3173-5p
154	34	-2.00575	323	10	-5.5468	hsa-miR-6751-5p
770	28	-4.60573	245	13	-4.77457	hsa-miR-190a-5p
384	5	-6.06015	109	8	-4.29713	hsa-miR-182-3p
245	22	-3.30046	100	9	-4.00593	hsa-miR-433-5p
537	12	-5.30026	107	12	-3.69428	hsa-miR-5587-3p
175	8	-4.26141	132	17	-3.49965	hsa-miR-7702
1195	296	-1.8431	858	117	-3.42699	hsa-miR-128-2-5p
634	52	-3.43511	1872	272	-3.33637	hsa-miR-616-5p
2608	72	-5.00669	2593	385	-3.30537	hsa-miR-4451
104	4	-4.49062	119	20	-3.11761	hsa-miR-5192
323	64	-2.1635	311	62	-2.87777	hsa-miR-8068
631	3	-7.49151	820	164	-2.87498	hsa-miR-132-3p
7039	1567	-1.99745	2408	531	-2.73489	hsa-miR-340-5p
1696	346	-2.12308	1144	260	-2.69099	hsa-miR-4637
165	5	-4.84217	96	22	-2.67152	hsa-miR-373-3p
2058	547	-1.74158	543	129	-2.62637	hsa-miR-3122
1143	11	-6.51416	226	55	-2.58975	hsa-miR-4499

150	15	-3.1421	134	34	-2.5276	hsa-miR-515-3p
4178	122	-4.92668	1262	322	-2.52421	hsa-miR-1307-5p
198	20	-3.1301	147	38	-2.50129	hsa-miR-301b-5p
9023	2145	-1.90274	1504	422	-2.38726	hsa-miR-193a-3p
375	64	-2.37877	447	128	-2.35697	hsa-miR-498
546	21	-4.5229	104	31	-2.29493	hsa-miR-4655-3p
11424	2938	-1.78929	1324	410	-2.24498	hsa-miR-32-5p
17906	3278	-2.27968	14688	4788	-2.17129	hsa-miR-19b-3p

Table 7.9 *Shared downregulated miRNAs in PC-3 and DU145 holoclones*

Log_2FC: Log_2 Fold Change. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells. RC refers to read count.

DPtM (RC)	DHM (RC)	log_2FC	PPtM (RC)	PHM (RC)	log_2FC	miRNA
0	133	10.13238	5	117	4.408844	hsa-miR-376b-5p
26	415	4.137498	52	272	2.277505	hsa-miR-628-5p
209	1517	3.006683	148	767	2.26583	hsa-miR-136-5p
16	127	3.126062	32	160	2.21081	hsa-miR-4687-5p

Table 7.10 *Shared upregulated miRNAs in PC-3 and DU145 holoclone-derived murine tumours.*

Log_2FC: Log_2 Fold Change. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour. RC refers to read count.

DPtM (RC)	DHM (RC)	log_2FC	PPtM (RC)	PHM (RC)	log_2FC	miRNA
163	3	-5.56116	300	0	-11.2838	hsa-miR-3174
99	12	-2.88429	474	8	-5.97279	hsa-miR-508-5p
100	20	-2.16746	275	5	-5.85193	hsa-miR-7109-3p
145	2	-5.95027	313	6	-5.78164	hsa-miR-6769a-3p
307	6	-5.50168	525	11	-5.66704	hsa-miR-3617-5p
188	19	-3.15086	287	6	-5.65658	hsa-miR-4302
226	30	-2.7606	696	18	-5.36979	hsa-miR-6813-3p
116	4	-4.66963	263	7	-5.31262	hsa-miR-33a-3p
205	48	-1.94405	344	10	-5.19313	hsa-miR-296-3p
184	2	-6.29365	453	16	-4.91898	hsa-miR-1233-5p
116	4	-4.66963	197	7	-4.89598	hsa-miR-4644
220	37	-2.42032	278	10	-4.88593	hsa-miR-4683
173	33	-2.23832	138	5	-4.8578	hsa-miR-1301-3p
336	13	-4.53151	738	30	-4.72147	hsa-miR-665
110	5	-4.2795	158	7	-4.57792	hsa-miR-5006-3p
111	12	-3.04914	437	20	-4.54751	hsa-miR-182-3p
202	27	-2.75011	696	34	-4.45711	hsa-miR-636
919	20	-5.36584	310	16	-4.37191	hsa-miR-381-3p
263	71	-1.73965	208	11	-4.33181	hsa-miR-23a-5p
161	4	-5.14211	131	7	-4.30779	hsa-miR-664a-3p
174	46	-1.76893	125	7	-4.24022	hsa-miR-6859-3p
233	12	-4.11802	138	8	-4.19346	hsa-miR-30b-3p
167	30	-2.32443	423	25	-4.18048	hsa-miR-6790-3p

121	27	-2.01139	431	26	-4.1512	hsa-miR-4734
115	1	-6.53761	174	12	-3.95035	hsa-miR-4764-5p
161	43	-1.75402	187	14	-3.83404	hsa-miR-6752-3p
368	99	-1.74516	1961	148	-3.83362	hsa-miR-4532
296	22	-3.59507	651	50	-3.80606	hsa-miR-3928-3p
86	16	-2.26998	598	48	-3.74231	hsa-miR-6889-5p
136	16	-2.93037	816	66	-3.73228	hsa-miR-6792-5p
388	80	-2.12851	1399	116	-3.69758	hsa-miR-940
240	53	-2.0287	738	63	-3.65434	hsa-miR-6765-3p
166	23	-2.69737	673	58	-3.64039	hsa-miR-2114-5p
152	12	-3.50219	531	50	-3.51217	hsa-miR-491-5p
154	16	-3.10953	279	28	-3.41759	hsa-miR-6132
246	32	-2.79012	323	37	-3.2283	hsa-miR-5585-3p
1243	207	-2.43762	3265	435	-3.01449	hsa-miR-663b
220	14	-3.81487	575	77	-3.00538	hsa-miR-6872-3p
728	111	-2.56427	2407	336	-2.94709	hsa-miR-6785-5p
174	32	-2.29086	293	41	-2.94011	hsa-miR-937-3p
541	80	-2.60794	1281	181	-2.92918	hsa-miR-3651
591	108	-2.30303	1310	190	-2.89151	hsa-miR-4292
198	26	-2.77548	145	22	-2.82007	hsa-miR-6068
90	20	-2.01567	747	114	-2.81754	hsa-miR-4674
214	23	-3.06354	484	76	-2.77571	hsa-miR-4801
499	83	-2.43835	1157	184	-2.75862	hsa-miR-4739
221	56	-1.83052	653	104	-2.75584	hsa-miR-4767
149	22	-2.60542	211	34	-2.73584	hsa-miR-1537-5p
152	9	-3.91253	245	41	-2.68211	hsa-miR-1265

1134	304	-1.75105	2210	371	-2.681	hsa-miR-4707-5p
1440	209	-2.63598	465	79	-2.66218	hsa-miR-424-3p
147	22	-2.58594	398	72	-2.57141	hsa-miR-323a-5p
191	12	-3.83144	94	17	-2.56488	hsa-miR-324-3p
264	62	-1.94033	754	140	-2.5349	hsa-miR-7108-5p
210	13	-3.85378	404	75	-2.5342	hsa-miR-6727-5p
112	21	-2.26077	452	85	-2.51584	hsa-miR-4726-5p
353	58	-2.4553	1963	374	-2.49841	hsa-miR-3607-3p
262	44	-2.423	879	168	-2.49336	hsa-miR-4690-5p
462	64	-2.70166	975	197	-2.41331	hsa-miR-7109-5p
222	12	-4.04829	97	20	-2.37732	hsa-miR-4256
452	104	-1.97068	328	69	-2.35372	hsa-miR-3940-3p
142	10	-3.66431	281	62	-2.2847	hsa-miR-619-5p
138	13	-3.24852	214	49	-2.23063	hsa-miR-6810-5p
397	83	-2.10854	1063	248	-2.20601	hsa-miR-5701
537	74	-2.70954	1025	240	-2.20078	hsa-miR-503-3p
216	36	-2.43326	734	175	-2.17446	hsa-miR-675-5p
147	13	-3.33959	82	20	-2.13529	hsa-miR-744-3p
530	87	-2.45747	729	179	-2.13202	hsa-miR-4284
1094	193	-2.3544	1449	363	-2.10349	hsa-miR-1180-3p
172	32	-2.2742	187	47	-2.09613	hsa-miR-8063
97	6	-3.84083	1081	274	-2.08647	hsa-miR-4286
215	57	-1.76536	497	127	-2.07416	hsa-miR-4533
122	10	-3.44552	147	38	-2.05488	hsa-miR-3139
241	3	-6.12495	108	28	-2.04935	hsa-miR-4268
321	65	-2.15419	451	123	-1.9802	hsa-miR-8074

352	71	-2.15998	886	242	-1.97859	hsa-miR-4638-3p
102	17	-2.42896	139	38	-1.97422	hsa-miR-766-5p
378	73	-2.22275	1289	360	-1.94667	hsa-miR-616-5p
387	43	-3.01861	764	222	-1.88927	hsa-miR-4789-3p
1654	329	-2.18156	1537	461	-1.84386	hsa-miR-331-3p
934	228	-1.88601	1545	466	-1.83579	hsa-miR-5787
1125	294	-1.78779	1198	367	-1.81328	hsa-miR-4787-5p
172	43	-1.8493	545	170	-1.78681	hsa-miR-4664-5p
296	36	-2.88759	698	225	-1.73959	hsa-miR-3129-3p
356	61	-2.3949	2824	919	-1.72633	hsa-miR-100-3p
131	28	-2.07359	373	122	-1.7181	hsa-miR-3939
428	99	-1.963	558	184	-1.70672	hsa-miR-6075

Table 7.11 *Shared downregulated miRNAs in PC-3 and DU145 holoclone-derived murine tumours.*

Log_2FC: Log_2 Fold Change. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour. RC refers to read count.

7.2.4 Analysis of miRNA:Target Interactions

Genes and miRNAs identified as being dysregulated were further investigated to identify interactions reconciling the two datasets. Databases of miRNA: target Interactions (MTIs) were queried to form lists of interacting miRNA-gene pairs, which in turn were used to create integrated networks. In total, five databases were interrogated. Three of these, miRWalk, miRTarBase and miRecords, include manually curated datasets of experimentally validated interactions. The remainder (TargetScan and miRTar) comprise lists of computationally predicted interactions, which have not yet been experimentally validated. The results from each separate database were concatenated and visualised as directed acyclic graphs (Figure 7.9, Figure 7.10, Figure 7.11). Full MTI network tables can be found in Appendix 2.

A definitive 'anti-correlation' was identified between interacting miRNA-gene pairs, which theoretically confirmed the efficacy of the sequencing run. For example, miR-27a, which was identified as downregulated in holoclones, was found to target KITLG and as previously mentioned this gene was identified as highly upregulated in holoclones. This analysis allows the visualisation of potentially critical regulatory networks, which are dysregulated in prostate cancer holoclones.

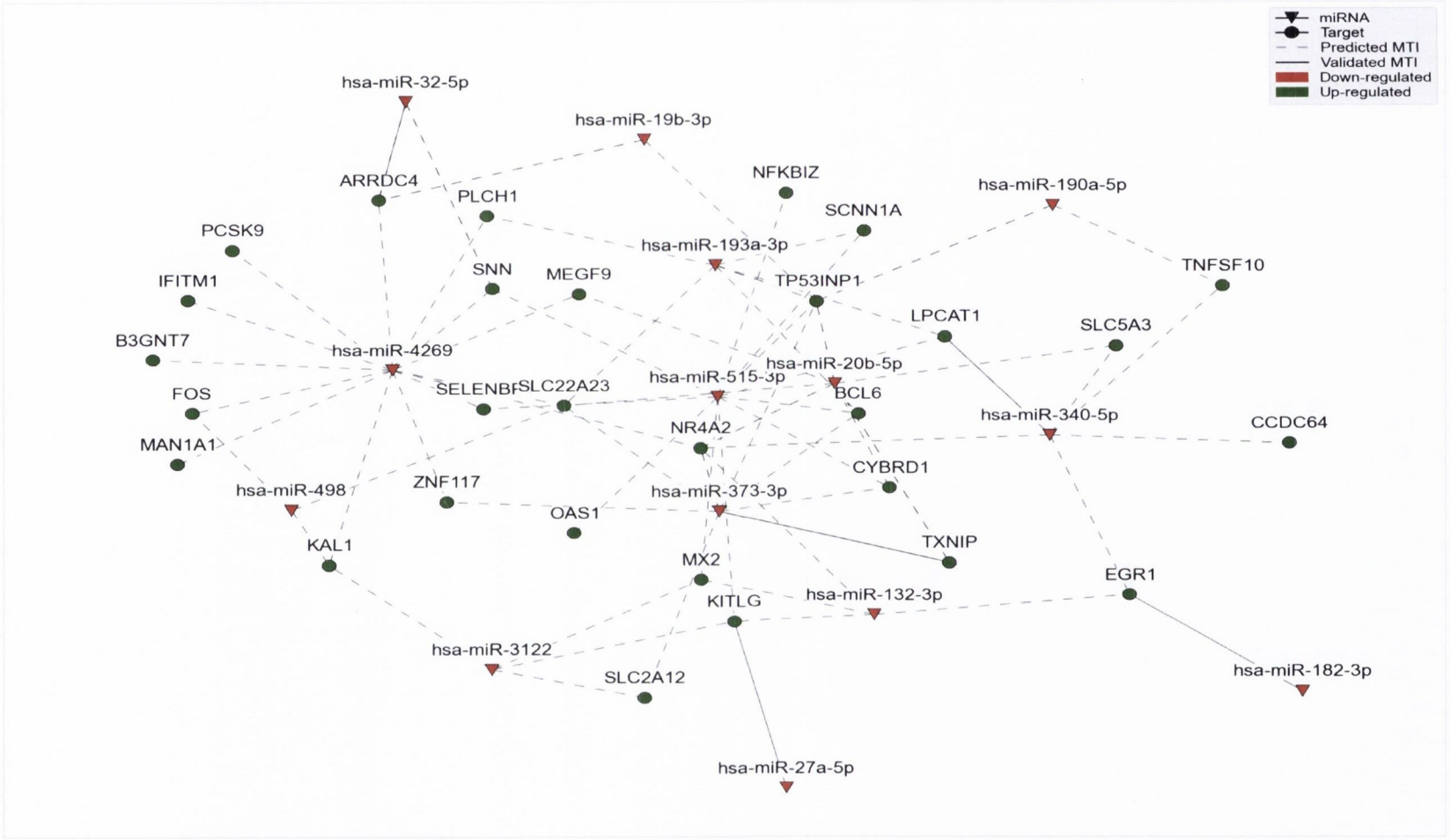

Figure 7.9 *Network of miRNA: target interactions (MTIs) for genes upregulated in holoclones compared to parental cells.*

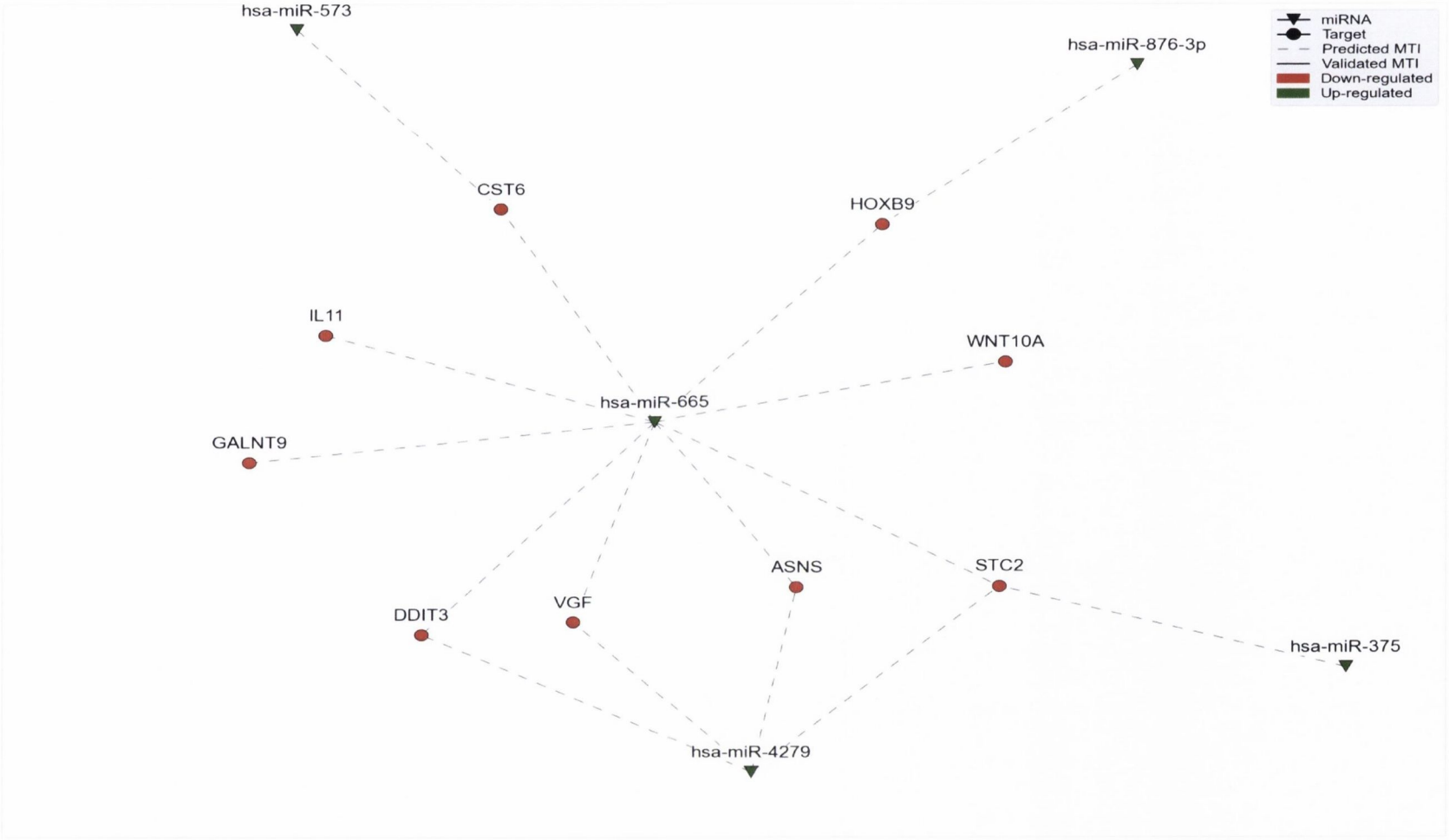

Figure 7.10 *Network of miRNA: target interactions (MTIs) for genes downregulated in holoclones compared to parental cells.*

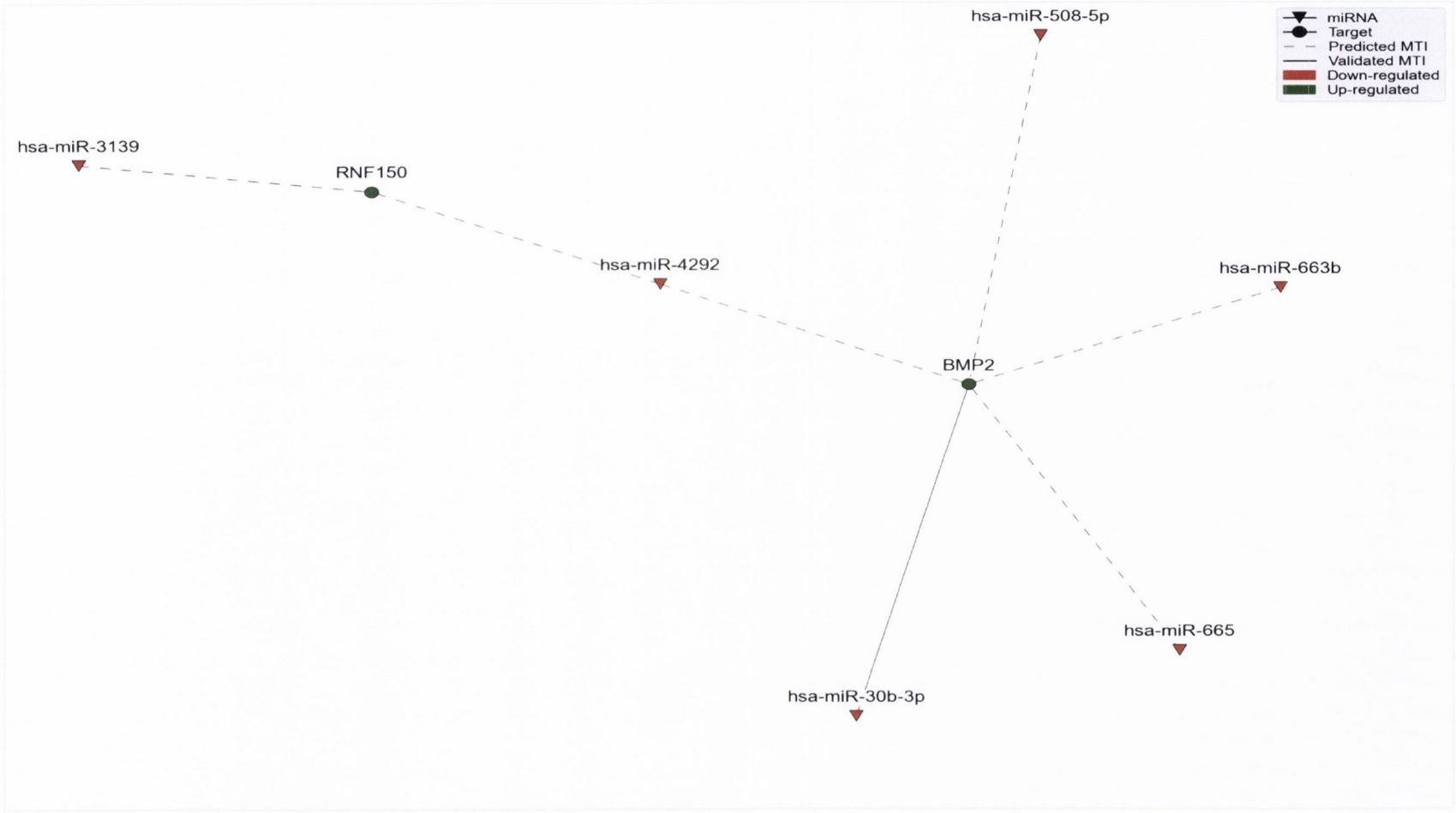

Figure 7.11 *Network of miRNA: target interactions (MTIs) for genes upregulated in holoclone-derived tumours compared to parental tumours.*

7.2.5 Long Non-coding RNA Profiles

Tophat was utilised to map reads to the Ensembl human reference genome (build GRCh38, release 76) and LNCipedia (LNCipedia.org). Transcripts corresponding to annotated long ncRNAs were identified (Trapnell *et al.*, 2009). Global changes in transcript abundance were elucidated as previously described (7.2.1).

Seven annotated long ncRNA transcripts were identified as differentially expressed in holoclones when compared to parental cells (PC-3 and DU145) (Figure 7.12). In addition, seven lncRNAs were also identified as differentially expressed in holoclone-derived murine tumours when compared to parental tumours (Figure 7.13). The majority of these ncRNAs are classified as novel lincRNAs in the Ensembl database (Table 7.12, Table 7.13, Table 7.14, Table 7.15).

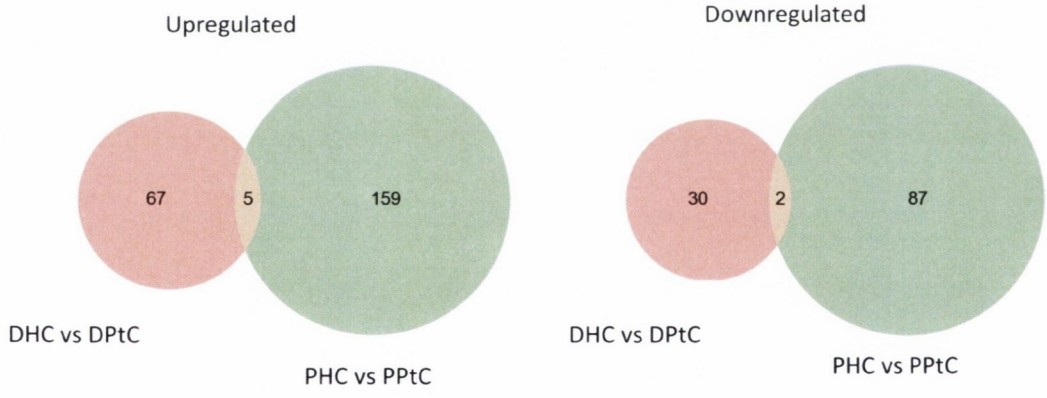

Figure 7.12 *Venn diagrams detailing shared and distinct lncRNA expression among DU145 and PC-3 parent and holoclone cellular samples.*

DHC: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells. RC refers to read count.

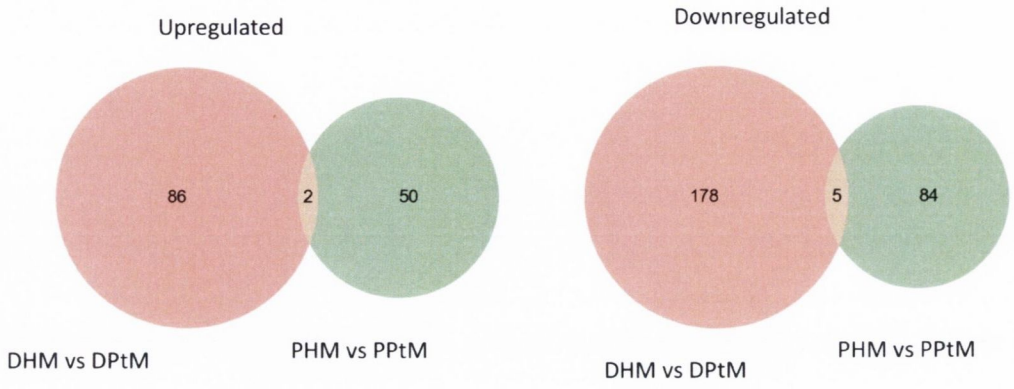

Figure 7.13 *Venn diagrams detailing shared and distinct lncRNA expression among DU145 and PC-3 parent and holoclone murine tumour samples.*

DHM: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour. RC refers to read count.

DPtC (RC)	DHC (RC)	log_2FC	PPtC (RC)	PHC (RC)	log_2FC	lncRNA Name	Annotation
29	531	4.050199	49	525	3.758018	AC138128.1	Novel lincRNA
31	138	2.011334	50	135	1.770471	linc-CBWD3-2	Novel lincRNA
2	31	3.738067	4	39	3.580317	HOXA11-AS	Antisense RNA
1	19	3.956808	7	39	2.793977	RP1-140K8.5	Novel lincRNA
8	73	3.032275	40	136	2.102034	RP1-193H18.2	Novel lincRNA

Table 7.12 *Annotated long ncRNAs upregulated in holoclones compared to parental cells (both PC-3 and DU145).*

Log_2FC: Log_2 Fold Change. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells. RC refers to read count

DPtM (RC)	DHM (RC)	log_2FC	PPtM (RC)	PHM (RC)	log_2FC	lncRNA Name	Annotation
25	2	-3.69801	37	4	-2.83528	LA16c-360H6.3	Antisense RNA
20	1	-4.29171	43	5	-2.73717	RP11-3L8.3	Novel lincRNA

Table 7.13 *Annotated long ncRNAs downregulated in holoclones compared to parental cells (both PC-3 and DU145).*

Log_2FC: Log_2 Fold Change. **DHC**: DU145 holoclone cells. **PHC**: PC-3 holoclone cells. **DPtC**: DU145 parental cells. **PPtC**: PC-3 parental cells. RC refers to read count

DPtM (RC)	DHM (RC)	log_2FC	PPtM (RC)	PHM (RC)	log_2FC	lncRNA Name	Annotation
2	17	3.060887	0	16	7.114962	linc-TLL1-1	Novel lincRNA
17	84	2.348239	2	23	3.639426	RP11-399O19.8	Novel lincRNA

Table 7.14 *Annotated long ncRNAs upregulated in holoclone-derived murine tumours compared to parental tumours.*

Log_2FC: Log_2 Fold Change. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour. RC refers to read count.

DPtM (RC)	DHM (RC)	log_2FC	PPtM (RC)	PHM (RC)	log_2FC	lncRNA Name	Annotation
108	30	-1.9254	93	15	-2.5487	RP4-740C4.6	Novel lincRNA
102	8	-3.7331	95	15	-2.5793	RP11-290F24.6	Novel lincRNA
238	0	-10.93713	101	1	-6.42036	CTD-2213F21.1	Novel lincRNA
12	1	-3.38111	15	0	-6.82224	linc-USP25-3	Novel lincRNA
79	19	-1.99685	42	8	-2.17412	NAV2-AS1	Antisense RNA

Table 7.15 *Annotated long ncRNAs down regulated in holoclone-derived murine tumours compared to parental tumours.*

Log_2FC: Log_2 Fold Change. **DHM**: DU145 holoclone murine tumour. **PHM**: PC-3 holoclone murine tumour. **DPtM**: DU145 parental murine tumour. **PPtM**: PC-3 parental murine tumour. RC refers to read count.

7.3 Discussion

The identification of a robust cancer stem cell population with validated markers for each tumour entity remains enigmatic. The development of simplistic cancer stem cell models has heralded a surge in the field of cancer stem cell marker identification. Much emphasis has been placed on the identification of universal surface markers of stemness across a variety of tumour types, their utility supported by the tumorigenic capacity of these isolates. However, in reality, natural biologic complexity has most likely complicated the identification of the correct components which uniquely define cancer stem cells. A provocative postulation, which in recent times has gained momentum, is that cancer stem cells do not represent a definitive subpopulation within a solid tumour but rather a transient, highly dynamic state which cells can cycle through over time. As controversial as this idea seems, there has been evidence to suggest that bulk tumour cells possess the intrinsic ability to transition to a stem-like state (Mikkelsen *et al.,* 2008). Indeed, this study has demonstrated that functionally and phenotypically plastic cells can be derived from differentiated, established cell lines. However, the driving force behind this dynamic equilibrium remains to be established. As do the constellation of molecular alterations which delineate these cells from the bulk population. Herein, we sought to exhaustively analyse the genetic composition of holoclones in order to identify not only the regulatory mechanisms underpinning cancer stem cell potential but also the differential gene expression patterns characteristic of this cellular population.

7.3.1 Identification of Candidate Genes Regulating Prostate Cancer Holoclone Identity

In recent years, RNA-Seq has begun to replace microarray technology as the standard for gene expression analysis due to its wider depth of coverage and relatively low level of technical variance compared to standard microarray technologies (Al Seesi *et al.,* 2014). However, the cost of RNA-Seq experiments remains high and as a result these experiments (as in this study) often have few or no biological replicates. As previously mentioned (7.2.1), using the raw fold change of the expression levels of a gene between two samples as a measure of differential expression can complicate the extrapolation of any hard conclusions as it is difficult to extract the most biologically relevant alterations. Hence, it was postulated that the differential gene expression patterns common to holoclones of both cell lines represented the most pertinent molecular alterations (Figure 7.2, Figure 7.3). While this method does not account for fragmentation or amplification variability in library preparation and sequencing, it does provide a descriptive analysis of the basic differential gene expression features, which may characterise prostate cancer stem cells. 41 genes were found to be commonly upregulated in PC-3 and DU145 holoclones when compared to their

differentiated parental counterpart. Most notably, a number of these genes have well-documented roles in the development and maintenance of human haematopoietic stem cells (Terskikh *et al.,* 2003).

EGR1

Early growth response 1 (EGR1) is a member of the immediate early response zinc-finger transcription factor family (Gashler and Sukhatme, 1995). EGR1, once bound to DNA, can act as a multifunctional transcriptional activator or repressor through cofactor-dependent interactions (Thiel and Cibelli, 2002). This gene has previously been reported to be overexpressed in prostate cancer tissue and has been specifically implicated in disease progression (Abdulkadir *et al.*, 2001). Transgenic mouse models of prostate carcinoma have elegantly demonstrated that EGR1 deficiency drastically delays the progression of prostatic intraepithelial neoplasia to invasive carcinoma suggesting that this transcription factor critically regulates genes necessary for prostate tumour progression (Abdulkadir *et al.,* 2001). EGR1 is a short-lived protein induced by a large number of extracellular stimuli including hypoxia, hyperoxia, chemotherapeutic agents and irradiation. A broad spectrum of cellular processes are regulated by this powerful transcription factor ranging from differentiation to transformation, growth to growth arrest and survival to apoptosis (Adamson *et al.,* 2003). However, most importantly, EGR1 has a well-documented stem cell association. The pronounced enrichment of EGR1 has been demonstrated in the most primitive subset of long-term reconstituting haematopoietic stem cells (HSCs) in wild-type bone marrow. This expression ameliorates as HSCs are induced to differentiate. Furthermore the absence of this protein in *egr1*$^{-/-}$ mice stimulates an increase in HSC proliferation and migration within the bone marrow, indicating that this protein is crucially involved in maintaining baseline HSC quiescence within the bone marrow niche (Min *et al.,* 2008). EGR1 expression has also been implicated in the expansion of neural stem cells and progenitors following hypoxic-ischemic brain injury (Alagappan *et al.,* 2013). Assuming the analogies, which have long been drawn between normal and cancer stem cells are accurate, it may be likely that EGR1 plays a similar role in the maintenance of prostate cancer stem cells, particularly given its previously reported association with disease progression. In support of this hypothesis, a recent study has demonstrated that pre-existing stem cell-like and neuroendocrine prostate cancer cells are selected by castration in the human prostate xenograft BM18 (Germann *et al.,* 2012). Furthermore, only the castration-resistant stem like-cells were capable of recapitulating BM18 tumour growth following reintroduction of androgen. Notably, EGR1 expression was enriched in these stem-like cells. These findings are highly significant given the postulation that the cancer stem cell niche represents the tumour-reinitiating subset in castration-resistant prostate cancer. Taken together, these

findings suggest that EGR1 may represent a previously unappreciated prostate cancer stem cell marker, particularly given its level of upregulation in prostate cancer holoclones (PC-3: 5-fold, DU145: 2.24-fold) (Table 7.1).

KITLG/SCF

KITLG (c-Kit ligand) otherwise known as Stem Cell Factor (SCF), is a dimeric cytokine that binds and activates the receptor tyrosine kinase c-Kit, resulting in autophosphorylation and activation of signal transduction (Lennartsson *et al.,* 2012). Signal transduction from this receptor mediates survival, migration and proliferation of early stem cell progeny. In particular, it has been demonstrated to function at multiple levels of haematopoiesis. *In vitro* murine experiments have demonstrated that SCF is a potent growth and survival factor for primitive haematopoietic cells. Studies have demonstrated that the administration of SCF to normal mice induces a 3-fold increase in the number of pluripotent haematopoietic stem cells in the peripheral blood and spleen of treated mice (Bodine *et al.,* 1993). This exquisite sensitivity to SCF has been replicated in a number of studies. Infact it has been demonstrated that highly purified long-term haematopoietic stem cells can only respond to three known cytokines in serum-free medium; TPO (thrombopoietin), IL-3 and SCF (Seita *et al.,* 2010). Point mutations which alter the function of the SCF protein are associated with a range of phenotypic anomalies including macrocytic anaemia, decreased fertility, abnormal melanogenesis and depletion of tissue mast cell numbers indicating that this cytokine plays a critical developmental role (Broudy, 1997). In addition to its developmental function, SCF is also constitutively expressed by adult marrow endothelial cells and fibroblasts indicating that this protein may also be necessary for normal basal haematopoiesis (Broudy, 1997). Excessive c-Kit signalling has been implicated in the development and progression of multiple cancer types including leukaemia, gastrointestinal tumours and prostate cancer (Wiesner *et al.,* 2008). Using an experimental model of prostate cancer bone metastasis, Wiesner *et al.,* (2008) found that intraosseous bone tumours formed by otherwise c-Kit-negative PC-3 cells, strongly expressed c-Kit and SCF. These findings suggest that the SCF-positive phenotype could be characteristic of stem-like cancer cells displaying an elevated metastatic potential. Given its powerful multi-lineage involvement, the observed upregulation of SCF in prostate cancer holoclones, is highly significant as this event may represent a signalling pathway critical to the activity of prostate cancer stem cells.

NR4A2

NR4A2 is a transcription factor belonging to the steroid nuclear hormone receptor superfamily, which has previously been implicated in cell transformation and the suppression of apoptosis in multiple cancer cell lines (Ke *et al.,* 2004). Similar to EGR1, the orphan

receptor subfamily of which NR4A2 is a member (known as the Nurr7 proteins), are immediate early genes induced by growth factors, serum and receptor engagement (Winoto *et al.,* 2002). Critically, this pro-survival gene has been identified in an 8-gene signature characteristic of bone and soft tissue cancer stem cells. Murase *et al.,* (2009) isolated a side population of cells from human bone sarcoma cell lines which possessed significant *in vivo* tumour-initiating ability. In addition, these cells could generate spherical colonies, which resulted in regeneration of both side population and main population cells indicating the self-renewal potential of this cellular subset. Microarray profiling of side population cells demonstrated that 23 genes were highly upregulated in these cells, 8 of which have previous tumorigenic associations within the literature (VPF, c20orf14, MCL1, IRX3, NRLP12, PTN, LMNA and NR4A2). Similar to EGR1 and KITLG, NR4A2 is a crucial molecular switch, which has been implicated in a plethora of physiologic pathways including apoptosis, DNA repair, proliferation, migration, inflammation, metabolism and angiogenesis (Mohan *et al.,* 2012). This gene has also been implicated in the maintenance of haematopoietic stem cell quiescence and a recent study has demonstrated that expression of this protein identifies a distinct population of highly primitive long-term haematopoietic stem cells (Land *et al.,* 2015).

TXNIP

Thioredoxin-interacting protein is a 50kDa protein belonging to the arrestin family. The TXNIP gene (also known as VDUP-1) was originally identified as a novel cDNA induced by the administration of 1,25-dihydroxyvitamin D3 for the treatment of leukaemia (Chen *et al.,* 1994). This protein is a regulator of a broad spectrum of cellular functions including maturation of natural killer cells, immune regulation, glucose and lipid metabolism, renal function, homeostasis and haematopoiesis (Jeong *et al.,* 2009). TXNIP has been demonstrated to function as a mediator of oxidative stress, through inhibiting the activity of the thioredoxin peptide or limiting its bioavailability. TXNIP also functions as a tumour and metastasis suppressor. Expression of this protein has been shown to be depleted in many tumour types and its overexpression inhibits proliferation through the stimulation of G_0/G_1 cell cycle arrest (Han *et al.,* 2003). However, most notably, TXNIP has been implicated as a potent modulator of haematopoietic stem cell function, particularly in the regulation of self-renewal capability. An elegant study by Jeong *et al.,* (2009) has demonstrated that TXNIP regulates HSC quiescence and migration under stress conditions. This study demonstrated that *Txnip*$^{-/-}$ mice have similar frequencies of HSCs as wild-type mice however; *Txnip*$^{-/-}$ HSCs display markedly decreased ability to regenerate following treatment with 5-fluorouracil and repeated transplantation. These findings indicate that HSC regulation is controlled by alternate mechanisms depending on steady-state/stress conditions and that TXNIP is a crucial regulator under stress conditions. The overexpression of this gene has

also been reported in prostate cancer stem-like cells derived from the DU145 cell line. Salvatori *et al.,* (2012) isolated a tumorigenic population of DU145 cells based upon expression of the antigenic profile $CD44^{+}/CD24^{-}$. This cellular population was found to generate highly vascularised, invasive tumours expressing low levels of E-cadherin and concomitantly high levels of vimentin. Expression analysis of these cellular isolates demonstrated differential expression of a number of genes including IGFBP3, KLF-4 and TXNIP. Interestingly, TXNIP was upregulated to almost the same degree in these tumour-initiating DU145 cells (5.48-fold) as our DU145 prostate cancer holoclones (5.62-fold). These findings indicate that the ability of TXNIP to regulate the self-renewal capacity of normal stem cells may also be applicable to the regulation of the prostate cancer stem cell population.

BCL6

BCL6 was first identified as a proto-oncogene in diffuse large B-cell lymphoma, a disease characterised by a high frequency of *BCL6-IGH* translocations (Ye *et al.,* 1995). BCL6-mediated transcriptional repression of p53 has since been demonstrated as a process critical for affinity maturation of B cells in germinal centres. In addition, this protein plays a fundamental role in pre-B cell survival (Duy *et al.,* 2010). In recent years, a number of studies have demonstrated the emerging role of BCL6 in *BCR-ABL1* driven leukaemias. For example, tyrosine kinase inhibitor (TKI) treatment of *BCR-ABL1*-transformed pre-B cell acute lymphoblastic leukaemia (ALL) cells has been demonstrated to induce BCL6 expression which allows these cells to persist following TKI treatment (Duy *et al.,* 2011). A study by Hurtz *et al.,* (2011) has demonstrated that the BCL-6 proto-oncogene is infact a critical downstream effector in self-renewal signalling of tumour-initiating chronic myeloid leukaemia (CML) cells. This study reported that the inhibition of BCL6 in human CML cells abrogates colony formation and diminishes leukaemia initiating ability in transplant recipients. BCL6 appears to maintain self-renewal ability in these cells through the transcriptional repression of p53 and Arf as blocking the formation of this complex has been found to suppress colony formation and drastically reduce the proliferative potential of these cells. These findings suggest that BCL6 function is an absolute requirement for tumour-initiation in CML and that the elevated expression levels of BCL6 in response to TKI treatment allows the long-term maintenance of a subset of treatment resistant tumorigenic stem-like cells. Our findings have demonstrated that BCL-6 transcript is markedly upregulated in PC-3 and DU145 holoclones indicating that a similar mechanism may be involved in the maintenance of self-renewal potential in prostate holoclones.

LPCAT1

Lysophosphatidylcholine (LPC) acyltransferase 1 (LPCAT1) is an enzyme crucial to the remodelling of phospholipids in the Lands' cycle (Lands, 1958). This protein has previously been demonstrated as overexpressed in colorectal adenocarcinoma when compared to normal rectal mucosa (Mansilla *et al.,* 2008). Prior to the characterisation of LPCAT1, increased enzymatic activity of LPC acyltransferase was demonstrated in human prostate carcinoma however the clinical significance of this anomaly remained unclear (Faas *et al.,* 2001). It has since been demonstrated that the preferential substrates of LPCAT1, LPC molecules, are markedly increased in tumorigenic prostate tissue compared to benign tissue suggesting a bias toward the production of phosphatidylcholine (PC). A relatively recent study has reported that LPCAT1 expression correlates with the progression of prostate cancer (Zhou *et al.,* 2012). Using immunohistochemical analysis, this study demonstrated that the expression levels of LPCAT1 are sufficient to differentiate prostate cancer tissue from benign prostatic hyperplasia, correlate to grade and stage and may be predictive of biochemical recurrence following radical prostatectomy indicating that LPCAT1 could possess clinical utility as an independent prognostic biomarker in prostate cancer. It has been postulated that abnormal LPCAT1 expression is associated with prostate cancer progression through modulating the production of unique phosphatidylcholine species, which may possess critical epigenetic function in addition to its canonical role in lipid synthesis. While the association of LPCAT1 with a cancer stem cell population has not been endorsed by previous literature, it is noteworthy that this gene was found to be upregulated in both PC-3 and DU145 holoclones. Perhaps elevated levels of distinct PC species as a result of LPCAT1 overexpression exert a pathologic effect through the epigenetic regulation of cancer stem cell gene expression. However, future expanded investigations will be required in order to establish a relationship between abnormal LPCAT1 expression and stem phenotype.

IL-24

The Interleukin-24 gene is a member of the IL-10 cytokine family. IL-24 is the only member of this family which has exhibited anti-tumour activity both *in vitro* and *in vivo* (Panneerselvam *et al.,* 2013). For example, loss of IL-24 expression has been significantly correlated with disease progression in multiple cancer types including lung and melanoma (Ishikawa *et al.,* 2005; Ellerhorst *et al.,* 2002). Ellerhorst *et al.,* (2002) also demonstrated an inverse correlation between IL-24 expression and tumour cell invasiveness in melanoma cell lines. Further studies have demonstrated that restoration of IL-24 protein expression abrogates tumour growth *in vitro* and *in vivo* (Su *et al.,* 1998). IL-24 expression has also been found to enhance the expression of E-cadherin; a protein crucially involved in cell-cell

adhesion which is downregulated during epithelial mesenchymal transition (as discussed in Chapter 6). Taken together these findings strongly implicate IL-24 as a potent anti-tumour cytokine. Our findings have demonstrated the common downregulation of IL-24 in PC-3 and DU145 holoclones (5.13-fold and 2.45-fold respectively). Thus, it may be postulated that the invasive phenotype characteristic of stem-like cells is mediated via the downregulation of this tumour suppressor signalling protein. In addition to the anti-metastatic capabilities of this protein, studies have demonstrated that the addition of IL-24 to human umbilical vein endothelial cells (HUVEC) results in a dose-dependent inhibition of endothelial cell proliferation and vascular endothelial growth factor (VEGF)-mediated migration indicating that IL-24 can also exert an anti-angiogenic effect (Ramesh *et al.,* 2003). As prostate cancer stem cells have been heavily implicated in the development of metastasis and angiogenesis, the observed downregulation of IL-24 in prostate holoclones provides an attractive insight into the potential mechanisms regulating this phenotype.

CLU

Clusterin is a heterodimeric disulfide-linked glycoprotein with a ubiquitous tissue distribution (Koltai, 2014). Seminal work by Sensibar *et al.,* (1995) was the first to elucidate the function of this highly conserved protein through the study of androgen-sensitive LNCaP cells. This study demonstrated that CLU was involved in protection against induced cell death by inhibiting the activity of tumour necrosis factor (TNF)-α in these cells. This work was the first to report that CLU overexpression can directly prevent cell death. In the intervening years, clusterin has been implicated in a diverse array of biological processes including sperm maturation, lipid transport, membrane recycling, cell adhesion and proliferation. Overexpression of clusterin has been demonstrated in multiple cancer types including breast, non-small cell lung, colorectal, ovary, bladder, renal and prostate (Higano *et al.,* 2013). In prostate cancer, overexpression of this protein has been associated with high Gleason score, more aggressive tumours and poorer outcomes (Steinberg *et al.,* 1997). Critically, clusterin expression has been shown to be elevated in patients following neoadjuvant hormone therapy for castration-resistant prostate cancer (July *et al.,* 2002). Furthermore, this protein is overexpressed in docetaxel resistant prostate cancer cell lines (Patterson *et al.,* 2006). Thus, clusterin plays a critical role in protecting cells from a variety of stressors that may induce apoptosis such as cytotoxic chemotherapy and androgen deprivation. CLU expression is low in most normal cells however its expression is potently stimulated by exogenous stressors. Therefore it could be hypothesised that the constitutive overexpression of this protein endows cells with an inherently resistant phenotype. The results of our sequencing analysis have demonstrated that clusterin is overexpressed in PC-3 and DU145 holoclones. As previously discussed, the failure of current conventional

therapies to eradicate cancer stem cells has been attributed to specific resistance mechanisms intrinsic in malignant cells possessing a stem phenotype. It may be postulated that the anti-apoptotic signalling pathway mediated by overexpression of clusterin represents a critical resistance mechanism employed by prostate cancer stem cells.

The identification of biologically distinct populations of "tumour-initiating" cells in malignancies of the haematopoietic system, brain, breast and prostate has altered our understanding of biology such that it is now widely accepted that stem cells have an indispensable role not only in the generation of complex multicellular organisms, but also in the conception and evolution of tumours. The capacity for self-renewal and the profound proliferative potential inherent in cells of type this are postulated to provide the potency necessary to drive the continued expansion of the malignant population. The observation that cancer stem cells remain viable despite the elimination of bulk tumour cells by standard chemotherapeutic agents has provided strength to the concept that this reservoir of drug-resistant cells are also the origin of tumour recurrence. This hypothesis has been further illustrated by the observation that less than 1% of disseminated tumour cells can generate clinically relevant micrometastases indicating that the inefficiency of metastasis can be attributed to the rarity of cancer stem cells (Luzzi *et al.,* 1998). The development of therapeutic strategies which selectively target cancer stem cells is a formidable objective. In order to deepen our understanding of cancer stem cells, it is imperative to define the physical features of these cells, which will not only permit their isolation but also differentiate them from normal stem cells. Herein, we sought to perform an extensive sequencing analysis of the mRNA transcripts, which characterise prostate cancer holoclones and differentiate them from their derivative parental cells. Our findings have demonstrated a unique gene signature, mutual to both PC-3 and DU145 holoclones, which comprises; multiple genes involved in the maintenance of self-renewal in primitive haematopoietic stem cells, genes which have been implicated in the progression of various cancer types and also a gene whose function in treatment resistance has been endorsed by a broad body of literature. Overall, these findings chronicle a cellular genotype defined by enhanced self-renewal potential, an elevated proliferative capacity, pro-metastatic and pro-angiogenic characteristics and a heightened resistant phenotype. This descriptive analysis has provided a snapshot of the molecular profile of prostate cancer holoclones. There are undoubtedly more genes, perhaps unique to each cell line, which may represent novel markers of stemness and also whose function in defining the stem phenotype remains ill-understood, thus a more comprehensive computational analysis of the RNA-seq data is justified.

7.3.2 Loss of Stem Signature *in vivo*

While a considerable number of genes were found to be differentially expressed between holoclone-derived murine tumours and their parental tumour counterpart, in total only 4 of these genes were common to both PC-3 and DU145 holoclone-derived tumours (Figure 7.3). Interestingly, no overlap was identified between these 4 genes and the 54 differentially expressed genes identified in cellular samples. These findings suggest a loss of intrinsic stem characteristics following engraftment into immune-deficient mice as cells differentiate in response to host micro-environmental cues. As cancer stem cells are promulgated as the cellular drivers of sub-clonal expansion, these findings are highly indicative of the growth and developmental potential inherent within this cellular subset (Rosen *et al.,* 2009). The observation that differential expression profiles demarcate holoclone-derived tumours from those generated by parental cells suggests the employment of disparate biological pathways *in vivo.* While it is beyond the scope of this thesis, the functional analysis of these pathways may provide an insight into the *in vivo* behaviour of prostate cancer stem cells.

7.3.3 Disparity in mRNA profiles between qPCR and RNA-Seq

Until recently, the analysis of mRNA expression in benign and tumour tissue has been conducted primarily using qRT-PCR. This technique has become one of the most widely used methods of gene quantification owing to its high specificity and sensitivity (Jozefczuk *et al.,* 2011). This methodology allows the generation of quantitative data across a wide range of biological matrices including fresh-frozen tissue, archival formalin-fixed specimens and tissue-cultured cells. qRT-PCR was utilised in this study to quantify the expression of stem-associated markers in PC-3 and DU145 holoclones. Our findings demonstrated the significant, reproducible upregulation of these genes particularly the stem cell markers NANOG, OCT4/POU5F1 and ALDH1 in prostate holoclones (Chapter 5). It was expected that the application of ultra-high-throughput RNA sequencing technology would reproduce these findings; however the expression levels of these genes were not found to be co-ordinately upregulated in holoclone samples (Appendix 2). While these findings were discouraging, one must be cognisant to the previously mentioned paucity of biological replicates in this study. Thus, while this data provides a descriptive analysis of potential molecular profiles, which characterise holoclones, ideally a statistical analysis is necessary to make unambiguous conclusions. Previous studies have demonstrated a high correlation in gene expression profiles generated by these platforms therefore it could be postulated that this discrepancy in expression profiles is a result of sample variation. The high degree of sensitivity inherent in qPCR meant that pooling of colonies (to yield appropriate RNA concentration) was not performed to the same extent as those required for RNA-Seq. The RNA-Seq experiment required approximately 5 µg of total RNA per sample. In order to

achieve this, holoclones were extensively pooled, which, in theory may have dampened certain transcript signals. In addition, it could be hypothesised that pooling of holoclones captured colonies at differential growth phases, whereby gene expression profiles may vary from holoclone subset to subset. In order to circumvent these issues, the incorporation of biological replicates into the study design would be desirable.

7.3.4 Differential miRNA Expression Profile Supports Invasive Phenotype of Holoclones

The relationship between microRNAs and cancer stem cells was perceived as early as 1993, when the first small non-coding RNAs were identified as dynamic regulators of development in *C. elegans* (Lee *et al.,* 1993). The regulatory function of miRNAs has since emerged as crucial in the establishment of cancer stem cell identity through the maintenance of a nucleus of transcription factor networks (Yu *et al.,* 2012). The implication of the pervasive role of miRNAs in cancer stem cell biology is that these small non-coding RNAs possess significant prospective therapeutic potential. In order to achieve novel improvements in existing diagnostic approaches, it will be necessary to identify miRNA signatures and characterise their cognate signalling pathways in cancer stem cells. Thus, we sought to examine not only the protein-coding genome of prostate cancer holoclones but also the small RNA species of these cellular colonies. Our findings have supported the ubiquitous role of microRNAs as regulators of cancer stem cell function. In total, 74 miRNAs have been identified as differentially expressed between parent and holoclone (common to PC-3 and DU145), some of which have already been heavily implicated in the regulation of cancer stem cells including; miR-128, miR-10b, miR-619 and miR-744. Additionally, miRNA: target interaction analysis has demonstrated an inverse relationship between these miRNAs and dysregulated gene pathways in holoclones. However, it is worthy to note, that there is a dearth of literature regarding a large proportion of the miRNAs identified, suggesting that these non-coding RNAs could represent novel markers, not only in terms of cancer stem cells, but also oncogenesis.

miR-128

A proteomics-based study by Khan *et al.,* (2010), which sought to investigate the molecular alterations associated with clinically aggressive prostate cancer, was the first study to indicate a role for miR-128 in prostate cancer progression. Levels of miR-128 were found to be elevated in benign prostate epithelial cell lines when compared to more invasive carcinoma cell lines. Furthermore, inhibition of miR-128 increased the invasive capacity of benign prostate epithelial cells, while overexpression of this microRNA abrogated invasion in prostate cancer cells. miR-128 has also been reported to be significantly downregulated in

malignant prostate tissue when compared to benign tissue suggesting that the loss of function of this miRNA plays a fundamental role in tumorigenesis (Ambs *et al.,* 2008).

A recent study has expanded upon these findings and demonstrated a compelling association between miR-128 and the modulation of prostate cancer stem cell-associated properties (Jin *et al.,* 2014). Initial transplantation assays demonstrated that PC-3, DU145 and PPC-1 cells transfected with miR-128 displayed a significantly diminished ability to establish tumours in NOD/SCID mice. miR-128 overexpression was also found to ameliorate prostate cancer cell growth and proliferation *in vitro.* Similar to previous reports, the invasive ability of these cells was also markedly reduced. Furthermore, overexpression of miR-128 drastically inhibited the ability of cell lines to generate holoclones (assessed using the colony forming assay technique). Indeed, expression of this microRNA was sufficient to induce a developmental shift to meroclone formation in DU145 cells. In addition, sphere-formation and clonogenicity of PC-3 and DU145 cells was significantly reduced. The pathogenic effects of miR-128 downregulation have also been described in breast cancer studies. Ectopic expression of miR-128 has been found to reduce the ability of breast cancer cells to generate mammospheres *in vitro* and inhibit the ability of these cells to generate tumours *in vivo.* Conversely, depletion of miR-128 was found to cause a significant increase in mammosphere formation (Qian *et al.,* 2012). Diminished levels of miR-128 in breast tumour tissue have also been associated with resistance to chemotherapeutic agents and poor survival rates (Zhu *et al.,* 2011). Thus, it would appear that the miR-128-mediated inhibition of cancer stem cell activity is not limited solely to prostate cancer.

miR-128 appears to exert these tumour suppressive effects through the regulation of several critical oncogenic and stem cell-associated targets namely; BMI1, NANOG and TGFBR1 (Godlewski *et al.,* 2008; Jin *et al.,* 2014). Taken together, these findings strongly indicate that miR-128 represents a potent negative regulator of stem/progenitor cells. Our findings have demonstrated that miR-128 is downregulated in both PC-3 and DU145 holoclones, consistent with their putative cancer stem cell-phenotype. The well-documented association between miR-128 and the regulation of cancer stem cell activities, not only in prostate cancer but in multiple tumour entities, indicates that miR-128 could represent a feasible therapeutic target.

miR-619

miR-619-5p is a 22 nucleotide small RNA encoded within an intron of the slingshot protein phosphatase 1 gene on human chromosome 12 (*SSH1*) (Ivashchenko *et al.,* 2014). Many of the predicted mRNA targets of this miRNA have been implicated in proliferation and apoptosis including; MAPK9, SLC6A2 and CRTC1. There is a dearth of literature describing

the pathological role of this miRNA in cancer; however a comprehensive molecular analysis of $CD34^+$ human haematopoietic stem-progenitor cells has demonstrated a role for this miRNA in the regulation of stem characteristics (Kim *et al.,* 2009). This expression analysis demonstrated that 45 microRNAs are present at high levels in the haematopoietic stem progenitor population. The most abundant of these miRNAs were identified as miR-566, miR-1273 and miR-619. The authors postulated that their high abundance infers a crucial function in the regulation of early haematopoiesis. The observed enrichment of miR-619 in PC-3 and DU145 holoclones is highly suggestive that this miRNA may play a fundamental role in the maintenance of the cancer stem cell population. The identification of a haematopoietic stem cell-like gene signature in holoclones provides strength to this concept as it may be postulated that cancer stem cells exploit physiologic signalling pathways analogous to those observed in early haematopoietic stem cells.

mir-10b

miR-10b has been extensively implicated in the development of metastasis in multiple tumour entities. It has previously been associated with high-grade gliomas, hepatocellular carcinoma and disseminated breast cancer (Sasayama *et al.,* 2009; Ladeiro *et al.,* 2008; Ma *et al.,* 2007). Microarray expression analysis has identified miR-10b as one of the most highly enriched microRNAs in breast cancer tissue (Zhang *et al.,* 2012). Furthermore, it has been demonstrated that overexpression of miR-10b enhances the invasive and metastatic potential of breast cancer cells *in vivo* (Ma *et al.,* 2010). miR-10b has also been identified as a target of the Twist transcription factor, which is highly expressed in metastatic breast cancer cells and stimulates tumour invasion *in vitro* and *in vivo* (Li *et al.,* 2013). Recently, a novel relationship has been established between miR-10b expression and the TGF-β1 signalling pathway (Han *et al.,* 2014). This study demonstrated that inhibition of miR-10b can ameliorate TGF-β1-mediated epithelial mesenchymal transition, proliferation and invasion in breast cancer cells. These findings provide robust evidence to advocate the role of miR-10b as a crucial molecular switch in the metastatic cascade. Interestingly, miR-10b expression has been demonstrated as lost in prostate tumour tissue when compared to benign, however expression of this miRNA has also been reported to be elevated in prostate cancer relapse patients compared to those who experience no relapse 3 years following radical prostatectomy (Walter *et al.,* 2013; Fendler *et al.,* 2011). Furthermore, Fendler *et al.,* (2011) have reported that miR-10b expression can strongly differentiate early- from late-biochemical relapse patients. In addition, none of the current clinicopathological parameters (Gleason score, PSA etc.) were capable of making this distinction indicating that miR-10b is an independent marker of biochemical relapse. The description of miR-10b in relation to tumour progression and metastasis in various malignancies including prostate cancer indicates that

this microRNA is crucially involved in the migration and invasion of malignant cells. Our study has demonstrated upregulation of miR-10b in PC-3 and DU145 holoclones compared to their differentiated parental counterpart. These findings are highly significant, given the multitude of studies which have promulgated cancer stem cells as the origin of metastases (Sun *et al.,* 2014). The precise mechanism of action of miR-10b has yet to be elucidated, however it could be postulated that this microRNA, and indeed others function as a molecular bridge between cancer stem cells and metastatic processes, such as epithelial mesenchymal transition.

miR-744

Huang *et al.,* (2010) were the first to report an association between miR-744 and prostate cancer. This study demonstrated that miR-744 can directly induce the expression of cyclin B1 (CCNB1) in mouse prostate adenocarcinoma cell lines. Conversely, knockdown of endogenous miR-744 was found to reduce CCNB1 levels indicating that this miRNA has a potent effect on cellular proliferation. miR-744 has also been identified as a pro-metastatic miRNA in a next-generation sequencing study, which investigated the small RNA repertoires of a metastatic and non-metastatic prostate xenograft cell line, both derived from one patient's primary cancer (Watahiki *et al.,* 2011). While there is no literature directly demonstrating the involvement of miR-744 in prostate cancer stem cell function, this miRNA has been implicated in the regulation of CSC characteristics derived from a colonic adenocarcinoma cell line. Zhang *et al.,* (2011) isolated a $CD133^+$ population of cells from the human HT29 colonic adenocarcinoma cell line, which displayed cancer stem-like characteristics. These cells were found to possess a heightened ability to generate spheres *in vitro* and an enhanced tumorigenic potential *in vivo.* In addition, they were found to highly express stem-associated genes including Oct3/4, Wnt2, BMI1, Notch1 and c-Myc. Microarray analysis of this cellular subset demonstrated the differential expression of 19 miRNAs, compared to $CD133^-$ cells. miR-744 was found to be upregulated in these colon cancer stem cells, indicating that this miRNA plays a role in the regulation of the stem phenotype. Similarly, miR-744 was identified as overexpressed in both PC-3 and DU145 holoclones. The observation that miR-744 is differentially expressed in metastatic prostate cancer is interesting, especially when one considers the mutual relationship which exists between cancer stem cells and the development of metastasis. Given the reported proliferative impact of miR-744 overexpression in prostate, it is highly likely that this miRNA functions in the development and maintenance of prostate cancer stem cells.

miR-340

miR-340 has been identified as downregulated in prostate tumours (Ambs *et al.,* 2008). There is no previous data implicating this miRNA in the regulation of prostate cancer stem cell activities, however there is evidence to suggest that the aberrant dysregulation of miR-340 is a feature of stem like-cells of various other malignancies. An intriguing study by Yamashita *et al.,* (2015) has demonstrated that miR-340 is significantly downregulated in human glioblastoma initiating cells (hGICs) when compared to human neural stem cells. Function analyses revealed that the expression of this miRNA suppressed hGIC proliferation, invasion and migration *in vitro* and drastically impaired the tumour-initiating ability of these cells in the mouse brain. Tissue plasminogen activator (PLAT) was also identified a direct target of this miRNA. These findings suggest that the downregulation of miR-340 contributes to malignant processes including proliferation and invasion via the modulation of cancer stem cell activity. Downregulation of miR-340 has also been implicated in the progression of breast cancer. Indeed, loss of miR-340 expression has been associated with lymph node metastasis, high-grade tumours, clinical stage, poor prognosis and increased expression of the oncoprotein c-Met (Wu *et al.,* 2011). The findings of our sequencing analysis have demonstrated that miR-340 is downregulated in both PC-3 and DU145 holoclones when compared to differentiated parental cells. This data is consistent with previous findings, which indicate that miR-340 downregulation is a feature of cancer stem cells. Thus, it may be hypothesised that the loss of expression of this miRNA facilitates the invasive phenotype of prostate cancer stem cells and ultimately correlates with the development of metastasis.

This sequencing analysis has provided ample evidence to advocate the belief that miRNA dysregulation is intimately associated with cancer stem cell identity and function. We have identified a miRNA signature of the prostate cancer stem cell phenotype comprising miRNAs whose role in CSC activities has been authenticated by previous literature and interestingly, a large proportion of miRNAs whose transcriptional targets and capacity in tumour perpetuation have yet to be elucidated. As previously discussed, despite the clinical significance of cancer stem cells, their regulation at the molecular level is relatively ambiguous. Thus, these novel miRNAs may be pivotal to deepening our understanding of the modulation of physiological and developmental pathways in prostate cancer stem cells. Functional genomics to investigate the impact of these miRNAs on the regulation of the stem cell phenotype is not only merited, but necessary to evaluate the downstream effect of their dysregulation. Realising the therapeutic potential of cancer stem cells is contingent on the identification of distinct biomolecular markers which discriminate these cells from the bulk tumour population and a full understanding of the 'miRNA-ome' of cancer stem cells will

undoubtedly answer many questions regarding the ontogeny and precise molecular features of this contentious cellular population.

7.3.5 Holoclones Preferentially Express Pro-metastatic miRNA Profile *in vivo*

Analysis of tumour specimens generated by PC-3 and DU145 holoclones *in vivo* has identified the concerted downregulation of multiple miRNAs, for which an analogous expression pattern has been implicated in prostate cancer metastasis including; miR-508, miR-33a, miR-331, miR-100, miR-503, and miR675.

For example miR-33a, miR-100 and miR-508 have all been identified as significantly downregulated in metastatic prostate cancer samples when compared to primary prostate tumours (Peng *et al.,* 2011). Furthermore, the expression of miR-33a has been found to inhibit lung cancer cell proliferation and invasion *in vitro* (Zhu *et al.,* 2014). The expression of miR-675 has been found to repress prostate cancer metastasis by targeting TGF-β1. This miRNA has also been demonstrated to be significantly downregulated in the metastatic prostate cancer cell line M12 compared to the non-metastatic prostate epithelial cell line P19 (Zhu *et al.,* 2014). The downregulation of miR-503 has been consistently identified in metastatic prostate cancer lesions while, miR-331 has been identified as downregulated in prostate tumour tissue compared to benign tissue (Watahiki *et al.,* 2011; Epis *et al.,* 2009). Finally and perhaps most interestingly, the loss of miR-100 has been demonstrated to play a pivotal role in prostate cancer metastasis. This miRNA has been consistently identified as downregulated in bone metastatic prostate cancer lesions compared to primary prostate cancer samples (Leite *et al.,* 2011). Moreover, the expression of miR-100 has been shown to negatively regulate migration, invasion, colony forming ability, epithelial mesenchymal transition, spheroid formation and the expression of stem-associated genes Klf4, c-Myc and OCT4 in PC-3 and DU145 cells. Furthermore, miR100 has been negatively correlated with bone metastasis in prostate cancer patients (Wang *et al.,* 2014). These findings indicate that miR-100 downregulation promotes the metastatic potential of prostate cancer cells specifically through the modulation of stemness. Thus, it may be postulated that prostate cancer stem cells can preferentially initiate a pro-metastatic expression pathway *in vivo.* Indeed, this postulation has been somewhat substantiated by the observation that holoclone-derived tumours express lower levels of E-cadherin, indicating a predilection for metastatic dissemination.

7.3.6 Altered Long Non-coding RNA Profiles of Prostate Cancer Holoclones

Given their abundance within the human genome, long non-coding RNAs were initially postulated as spurious transcriptional artefacts stemming from RNA polymerase infidelity

(Mercer *et al.,* 2009). However, it is now widely accepted that long non-coding RNAs represent relatively uncharacterised modulators of biological function, although it is difficult to infer their precise function from sequence and structure alone as lncRNAs typically exhibit a low degree of sequence conservation (Mercer *et al.,* 2009). In addition, the annotation of long non-coding RNAs has proven difficult as parsing of coding and non-coding transcripts is often complicated by the chance occurrence of open reading frames (ORF) in long non-coding sequences. In eukaryotes, protein-coding transcripts are conventionally defined by the presence of an ORF greater than 100 amino acids. However, many characterised lncRNAs are known to contain analogous ORFs. Despite improvements in the annotation of lncRNAs in recent years, there are many enigmatic transcripts, which exhibit both coding and non-coding attributes (Mercer *et al.,* 2009). Extensive overlapping of alternatively spliced coding and non-coding isoforms further confounds this issue. Indeed, many mRNAs can impart function at the RNA level. For example, *SRA,* the most unequivocally characterised lncRNA has been demonstrated to encode a protein, which induces a negative feedback loop with this ncRNA (Chooniedass-Kothari *et al.*, 2004). Nevertheless, increasing evidence is corroborating the cell type-specific context of ncRNAs and substantiating the role of these key molecules in human malignancy. Recent advancements in RNA-seq technology have permitted the delineation of transcriptional anomalies in cancer, including novel non-coding transcripts, which previously could not be quantified by conventional technologies. Although still largely uncharted, interactions between lincRNAs and known cancer-associated genes have been described (Cheetham *et al.,* 2013). Herein, we applied transcriptome sequencing to PC-3 and DU145 parental and holoclone samples (cellular- and murine tumour-derived) in an effort to identify prostate cancer stem cell-associated long non-coding RNAs.

Interestingly, only a small degree of overlap in annotated long non-coding transcripts was identified between parent and holoclone samples irrespective of cell line origin. This finding strengthens the assertion that long non-coding RNAs are exquisitely cell-type specific. Five lncRNAs were identified as upregulated in holoclones when compared to parental cells, while 2 were identified as downregulated. In addition, 2 lncRNAs were found to be upregulated in holoclone-derived tumours (compared to parental cell tumours), while 5 were found to be downregulated. According to the Ensembl database, these lncRNAs are predominantly annotated as novel lincRNAs. However, it must be noted, that the RNA-seq data generated by this study likely contains an array of unannotated long ncRNA species; both common and unique to holoclones originating from each cell line. Thus, in order to identify these novel transcripts, extensive computational and functional analysis is required.

As previously mentioned, thousands of large intergenic ncRNAs (lincRNAs) have been identified in mammals (7.1.1). However, the majority of these have yet to be functionally

characterised, which has precipitated a significant degree of contention regarding their biological role (Guttman *et al.,* 2011). In recent years, studies have begun to address this scarcity of knowledge through loss-of-function experiments and subsequent analysis of gene expression profiles. Interestingly, lincRNAs have emerged as profound regulators of pluripotency and differentiation. Knockdown of the most predominant lincRNAs expressed in mouse embryonic stem cells (ESCs) has been demonstrated to induce considerable alterations to the gene expression profiles of these cells; namely downregulation of endogenous levels of NANOG and OCT4. Furthermore, loss of expression of these lincRNAs was sufficient to ameliorate pluripotentiality. In addition, many ESC-associated lincRNAs have been shown to be directly regulated by critical pluripotency transcription factors and form interactions with chromatin proteins. LincRNAs were also identified to directly repress lineage-differentiation programs (Guttman *et al.,* 2011). These findings indicate that lincRNAs orchestrate a critical regulatory network, which maintains pluripotency in embryonic stem cells. It is postulated that the interaction of lincRNAs with ubiquitous regulatory protein complexes modulates cell-type specific gene expression patterns. Given the analogies drawn between non-malignant and cancer stem cells, it could be hypothesised that lincRNAs adopt a similar role as 'flexible scaffolds' in the maintenance of the cancer stem cell phenotype (Zappulla *et al.,* 2004). This hypothesis also provides an attractive explanation for the uneven sequence conservation observed in lincRNAs; the more conserved regions could represent protein-complex interaction sites. Testing this hypothesis will require extensive investigation, including identifying the cognate protein-complexes of specific lincRNAs identified in this study and determining the functional contribution of these interactions in terms of the gene expression profiles of prostate cancer stem cells.

7.4 Conclusion

The inception of the cancer stem cell hypothesis has engendered a fervent quest to identify biologic pathways that are active in cancer stem cells but disengaged in normal, differentiated cells. It has been postulated that the identification of prostate cancer stem cells offers a unique aperture for the development of selective therapies but exploitation of this avenue requires a refinement of the specific markers and pathways, which characterise prostate cancer stem cells. In this study, we have utilised a hypothesis-neutral next-generation sequencing approach to perform a global transcriptomic analysis of stem-like cells and their derivative tumour xenografts. We have identified a putative gene expression signature of prostate cancer holoclones, which depicts a pro-metastatic, pro-angiogenic, inherently resistant phenotype mediated by the expression of multiple genes including; SCF, EGR1, BCL6, IL-24 and LPCAT1. In addition, we have identified the complex regulatory circuitry instigating these gene expression patterns through a computational miRNA: target

interaction analysis. We have also identified novel lincRNAs, whose putative 'molecular scaffold' function may prove critical to the maintenance of prostate cancer stem cell identity. To our knowledge, this is the first study to perform an exhaustive genetic analysis of putative prostate cancer stem cells. Thus, we have identified genes and miRNAs whose role in the prostate cancer stem cell phenotype was previously unheralded. The wealth of data generated by this work, in terms of novel genes and non-coding RNA species will likely aid in the expansion of this area of study.

Discussion and Future Directions

Chapter 8

Chapter 8. Discussion and Future Directions

8.1 Introduction

Manifestations of prostate cancer vary widely, ranging from localised, indolent tumours to systemic hormone-refractory metastases. The highly heterogeneous nature of this malignancy has precipitated a significant degree of overtreatment. This clinical burden has caused fresh controversy in recent years and renewed the requirement to elucidate the pathological landscape of features associated with disease progression. A large body of literature supports the ability of multiple biomolecular markers to predict patient outcome, however at present none of these markers are used to guide clinical decision-making (Makarov *et al.,* 2008). A number of critical limitations are responsible for this; firstly the pathobiology of prostate cancer remains largely enigmatic, and secondly the multifocal and multiclonal heterogeneity of primary tumours precludes molecular taxonomic subclassification. Recent studies have documented the potential utility of integrated translational approaches to determine the evolving spectrum of molecular anomalies associated with disease behaviour and progression (Haffner *et al.,* 2013). This project focussed on identifying the differential miRNA and mRNA expression patterns, which may aid in the classification of lethal prostate cancer. This work formed the genomic portion of the Prostate Cancer Research Consortium's (PCRC) technologically integrated approach to identify the spectrum of molecular alterations which demarcate progressive disease.

In tandem with this genomic exploration of human clinical specimens, we sought to devise a cell line model representative of the functional plasticity believed to define the cancer stem cell population. In recent years, the identification of cancer stem cells in haematopoietic and solid tumours has induced a paradigm shift regarding tumour growth and disease progression, particularly in terms of prostate cancer. The existence of a prostate cancer stem cell with extensive replicative potential, offers an attractive explanation for the clonality of this malignancy. While the characteristics of cancer stem cells remain contentious, the clinical relevance of this population is endorsed by the finding that stem-associated gene expression signatures are predictive of patient outcome in several solid tumour types (Greaves *et al.,* 2011). The idea that self-renewing cancer stem cells drive and sustain clonal evolution suggests that the restraint of this population should be the fundamental goal of all therapeutic strategies.

Chapter 3 introduced expression profiling in prostate cancer through the description of a miRNA and proteomic expression analysis of a small, archival radical prostatectomy cohort. This study identified a number of difficulties associated with the quantification of expressional changes in clinical specimens, which may be exacerbated by the molecular

and cellular heterogeneity of prostate tumours. In addition, immunohistochemical analysis of a tissue microarray comprising this archival cohort replicated the findings of previous studies, which support the clinical potential of Ki-67 as a prognostic marker in prostate cancer. The significance of this finding and the reasons behind the relatively limited use of this marker in a clinical setting are discussed. Chapter 4 described the expression analysis of a gene/miRNA panel in a defined radical prostatectomy PCRC cohort comprising putatively indolent, significant and aggressive prostate carcinoma. This chapter discussed the pathological implication of an altered molecular genetic profile in putatively aggressive cases and explored the potential impact of this data longitudinally, as the cohort evolves and matures in the coming years.

The utility of prostate cancer cell lines as a surrogate source of cancer stem-like cells was demonstrated in Chapter 5. This chapter illustrated the contention surrounding ambivalent markers of stemness as multiple techniques were explored in an attempt to prospectively isolate prostate cancer stem cells. Colony forming assay, a technique involving single cell propagation was identified as the most robust method to generate prostate cancer holoclones, whose functional potential is postulated to represent that of cancer stem cells. The tumour-forming potential of the prostate cancer holoclones was investigated in Chapter 6 through a series of murine xenotransplantation assays. Our findings suggested that holoclones may possess an enhanced metastatic potential as demonstrated by their ability to modulate E-cadherin expression. Histopathologically, tumours generated by holoclone cells were found to be very similar to those generated by differentiated parental cells indicating the propensity of holoclones to generate 'histocopy' tumours. However, disparate *in vivo* tumour-forming dynamics and differential stem expression profiles in derivative holoclone-generated tumours raised the question of phenotypic and functional heterogeneity within the cancer stem cell population.

In order to elucidate the myriad of transcriptional alterations which accrue as differentiated parental cells transition to the holoclone phenotype during clonal propagation, an exhaustive genetic analysis of small ncRNA, long ncRNA and mRNA gene transcripts was performed, the results of which are discussed in the culminating Chapter of this thesis.

8.2 The Prostate Cancer Research Consortium's Integrated Analysis; a Novel Study

The Prostate Cancer Research Consortium biomarker discovery initiative is an ongoing novel study, which aims to correlate molecular markers quantified in a spectrum of biological matrices with the progression of prostate cancer. The fundamental objective of this study is to identify a clinically relevant, multi-class, predictive model capable of reliably classifying the molecular features of the aggressive disease subtype. No study has ever attempted to

perform an integrated analysis of this nature. To date, multiple technologies have been employed to probe the mRNA/miRNA expression profiles, hypermethylation landscape and the proteomic repertoires of the same cohort of treatment-naive radical prostatectomy specimens across multiple clinical institutions. In addition, cutting edge pathological image analysis has been employed in an attempt to reconcile molecular anomalies with the histopathological features present within these tumour specimens. The clinical samples, which fuelled this integrated analysis were drawn from the PCRC bioresource and subdivided into three categories postulated to best represent the biological continuum of this disease; indolent (Gleason score < 7, organ-confined), significant (Gleason pattern 4 present, organ-confined) and aggressive (Gleason pattern 4 or 5 present, non organ-confined). In the coming years, as patients within this well-defined cohort begin to experience biochemical recurrence and disease relapse, it will become apparent whether this integrated approach has the potential to inform clinical decision-making.

One of the hypotheses central to this thesis in particular, was that a differential mRNA/miRNA expression pattern characterises aggressive prostate carcinoma and that this unique expression signature may be harnessed to aid in the delineation of clinically indolent from lethal, aggressive disease. This study sought to overcome some of the issues which have restricted previous biomarker studies; namely unnecessarily large probe panels and the use of suboptimal sample sets where patients within a cohort are not matched to age, grade and preoperative PSA levels. The gene/miRNA panel for interrogation in the PCRC cohort was compiled through an exhaustive meta-analysis of the literature in order to identify the most cogent and biologically relevant targets. Subsequently, the gene panel was further refined by the construction of a correlation matrix, which identified genes possessing a known miRNA: target interaction (MTI) with one or more miRNAs found within our panel and an independent panel generated by Harvard collaborators (Chapter 4; 4.2.2).

The results of this large-scale expression analysis demonstrated that six genes were uniquely overexpressed in the aggressive patient subgroup; IGFBP3, SFRP4, CCNB1, FAM49B, AMACR, and MUC1. As discussed previously, the observed aberrant expression of these genes in tumorigenic tissue is not entirely surprising given their strong disease associations; however it is highly encouraging that their abnormal expression levels appear to statistically significantly differentiate the aggressive disease group from both the indolent and significant subgroups. In contrast to the mRNA analysis, miRNA expression profiling demonstrated that no miRNA candidate within the panel was significantly differentially expressed across all three disease subgroups. Given the well-documented ability of differential miRNA expression to classify prostate cancer subgroups, these findings were somewhat unexpected (Ren *et al.,* 2014). Even more so, considering the observed

dysregulation of mRNA transcripts, which are direct targets of miRNAs within the panel investigated. However, as previously mentioned, recent studies which describe the co-evolution of an altered stromal environment as cancer progresses have suggested that phenotypic and genotypic alterations which characterise this 'reactive stroma' microenvironment are infact key players in the genesis and progression of cancer (Barron *et al.,* 2012). Thus, it may be hypothesised that a deeper understanding of; a) the complex interrelationship which exists between reactive stromal cells and b) the transcriptomic alterations which define the tumour-driving potential of the reactive stroma, is necessary in order to accurately distinguish prostate cancer subtypes. Indeed, the downregulation of let-7c in prostate cancer stromal cells has been demonstrated to be significantly associated with the presence of extraprostatic extension (Ren *et al.,* 2014). This finding provides strength to the concept that disease-specific molecular alterations may be identified in tumour-associated stromal cells. In addition, it is prudent to note, that despite the paucity of a statistically significant differential miRNA expression pattern, it is postulated that the miRNA component of this study will still be of utility when incorporated into a multiparametric prediction model. Indeed, preliminary statistical analyses have demonstrated that collectively the integrated mRNA/miRNA panel can discriminate indolent from aggressive subgroups with dramatically high sensitivity and specificity (data not shown).

When scrutinised in isolation, the findings of this expression analysis are relatively conventional, however it is postulated that the strength of this study will become apparent when this data is merged with proteomic, hypermethylation and glycosylation data generated by the various components of the PCRC initiative. Gene and miRNA targets interrogated in this study were carefully chosen based upon previous reproducible disease associations in order to maximise the likelihood of identifying a cogent signature of aggressive disease and it is hoped that the combination of this signature with the various other multi-matrix markers will create a statistically powerful, accurate prognostic model.

8.2.1 Future Work: Validation of Prostate Cancer Research Consortium Biomarker Panel

Validation of the final PCRC biomarker panel in an independent cohort will be necessary in order to translate a potentially prognostic signature into a clinically feasible test. Should the final panel demonstrate utility in predicting prostate cancer progression and patient outcome in the PCRC cohort, this must be replicated in an additional cohort to ensure biomarker data is accurate, reliable and 'fit-for-purpose'. Thus, perhaps the most high-throughput approach would be the immunohistochemical analysis of targets in a tissue microarray of an independent cohort with full histopathological and clinical long-term follow up data.

8.2.2 Future Work: Analysis of mRNA/miRNA in Urinary Exosomes

The concept of analysing urinary exosomes to garner information regarding the pathophysiological state of their epithelial cells of origin has gained momentum in recent years (Nilsson *et al.,* 2009). Indeed, the observation that exosome secretion is elevated in malignancy effusions, serum and urine from cancer patients has provided circumstantial evidence to support the postulation that analysing the transcriptome of secreted exosomes in prostate cancer may be informative as to the overall disease status (Mitchell *et al.,* 2009). Hence, examination of the integrated mRNA/miRNA panel in urinary exosomes derived from the same PCRC patient cohort is a worthy endeavour. Exosomes should be isolated from biobank urine samples using ultracentrifugation and total RNA extracted for transcriptomic analysis. The identification of a concordant expression signature would corroborate the putative utility of exosomal analysis as a minimally invasive prognostic test.

8.3 The Genetic Fingerprint of Cancer Stem Cells

Although the cancer stem cell hypothesis has been reported to be a universal feature of all malignancies, the concept remains contentious. To date, there has been no unanimity on whether cancer stem cells are rare or abundant cells, or even whether they possess a definitive, consistent phenotype. However, the variability within the clonal architecture of cancer between individuals would suggest that these cells are more likely to be transitory entities (Greaves *et al.,* 2011). It is also argued that 'stemness' is inducible by certain micro-environmental conditions, such as hypoxic stress. The findings of this study would certainly lend support to this concept, as it has been demonstrated that functionally plastic cells expressing canonical stem markers can be sequestered from long-term established, differentiated cancer cell lines by single cell propagation (Chapter 5; 5.2.5). It is postulated that the sole constant phenotypic feature of cancer stem cells, among a background of genotypic diversity is the ability to self-renew, suggesting that the quantification of self-renewal activity through specific gene-expression signatures could be used to predict the clinical course of multiple cancer types. Thus, the identification of robust markers of stem-cell activity is of immense clinical importance. This study has utilised an assay predicated on the basis of self-renewal potential to induce colonies enriched for cancer stem cell properties. These colonies appear to fulfil the most feasibly stringent criteria for the identification of stem-like cells; self-renewal potential, multi-lineage differentiation capacity and tumour-initiation ability *in vivo,* which confirms the feasibility of this assay as a surrogate technique for the generation of cancer stem cells (Chapter 5; 5.2.7, Chapter 6). Indeed, the genetic heterogeneity of these cells was also inferred by the inconsistency in their expression profiles following xenotransplantation and the observed diversity in *in vivo* tumour-forming behaviour between cell lines (Chapter 6; 6.2.2.3, Chapter 7; 7.2.1).

Irrespective of the genetic divergence present among malignant stem-like cells, as indicated by initial literature, the identification of related genotypes is vital to cancer stem-cell restraint (Notta *et al.,* 2011; Clappier *et al.,* 2011).

This study sought to identify shared genetic components of cancer stem cells, which discriminate them from the bulk differentiated cells but unite them in the common properties of self-renewal, enhanced invasive capacity and treatment resistance. The identification of genes uniquely expressed in cancer stem cells is paramount to the development of novel targeted therapies. Next-generation sequencing analysis has identified a rich spectrum of genes and miRNAs whose expression is altered as these cells deviate to a stem-like state (Chapter 7). Many of the genes identified are multi-functional, potent transcriptional regulators indicating the pervasive dysregulation of signalling pathways in cancer stem cells. The identification of a gene signature which is indicative of haematopoietic stem cell identity is likely a result of the abundance of literature on this topic. The haematopoietic stem cell is the common ancestor of all blood cell types and is the most well characterised stem cell type in the human body (Kondo *et al.,* 2003). It is conceivable that the pathways exploited by these lineage-specific stem cells are analogous to those whose pathogenic dysregulation allows cancer stem cells to escape the restrictions imposed by the stem cell niche and drive tumour progression. In addition, many genes were identified whose role in the stem cell phenotype remains unclear, thus they warrant further investigation.

The observation that miRNA regulation plays a fundamental role in defining the features of cancer stem cells is somewhat of an antiquity. Multiple studies have reported dysregulated miRNA patterns, which contribute to the development of malignancy via the promotion of cancer stem cell properties; however the precise role of these miRNA expression patterns in stem cell function remains to be fully understood. In particular, the miRNA expression profiles of prostate cancer stem cells have yet to be described in great detail. The next-generation sequencing analysis of holoclones has provided ample circumstantial evidence to confirm the role of multiple miRNAs, which have previously been implicated in the regulation of cancer stem cells including; miR-128, miR-10, miR-619, miR-744 and miR-100. In addition, the PCRC expression analysis has demonstrated a significant dysregulation of stem-associated miRNAs in putatively aggressive disease. miRNA: target interaction (MTI) analysis has provided a visual representation of how the concerted activity of these miRNAs regulates a profusion of downstream physiologic pathways. The interrelationship between miRNAs and their cognate mRNA targets appears discordant and chaotic, which indicates that we are only beginning to comprehend the complexity of the genetic mechanisms underpinning 'stemness' in human malignancy.

Most notably, this sequencing study has identified a plethora of miRNAs for which there are no validated gene targets or associated literature, indicating that these miRNAs may represent novel and critical markers of prostate cancer stem cell potential. Hence, extensive investigation into the altered molecular mechanisms induced by the dysregulation of these novel miRNAs is required, in order to truly appreciate their role in cancer stem cell function.

8.3.1 Future Work: Serial Xenotransplantation

In order to conclusively demonstrate the self-renewal potential of prostate cancer holoclones, serial transplantation assays in NOD/SCID mice should be performed. Cells should be isolated from the initial tumour and grafted into further recipient animals. The formation of serially transplantable phenocopy tumours over multiple generations will definitively confirm the self-renewal potential of prostate holoclones. Furthermore cells could be isolated from transplanted tumours, cultured *in vitro* and resubmitted to colony forming assay to confirm that these cells retain the ability to recapitulate all colony morphologies to similar proportions as the original parental cell line.

8.3.2 Future Work: Functional Genomics of Stem-associated Genes

This study has demonstrated the upregulation of multiple genes, which have previously been associated with stem potential (EGR1, TXNIP, KITLG and LPCAT1). However many genes have been identified as differentially expressed in holoclones, whose function in 'stemness' remains unclear (e.g. KAL1, OAS1 and SNN). Conditional *in vitro* experiments could be performed to further elucidate the function of these genes in relation to prostate cancer stem cell behaviour. RNAi-mediated knockdown, followed by Western blotting to confirm knockdown could be performed in PC-3 and DU145 cells. Proliferation, clonogenicity, migration and invasion assays could be performed to determine whether knockdown of these genes ameliorates the induction of typical stem characteristics.

8.3.3 Future Work: Functional Analysis of Novel Stem-associated miRNAs

As previously mentioned, this study has identified a network of miRNAs differentially expressed between parent and holoclone samples, for which there are no predicted/validated targets (examined using the miRWalk, miRTarBase and miRecords databases) or previous literature, indicating that the discovery of these miRNAs is a relatively nascent event. For example, miR-6765 emerged as the most highly upregulated miRNA in PC-3 holoclones, while miR-4706 was identified as the most highly upregulated miRNA in DU145 holoclones (Chapter 7). It is impossible to discuss the potential significance of these dysregulated miRNAs as their function remains unknown. An *in silico* approach could be adopted whereby target prediction algorithms are utilised to identify

potential mRNA targets based upon seed region complementarity. Experimental validation of putative gene targets could then be performed using qRT-PCR, luciferase reporter assays and western blotting (Thomson *et al.,* 2011). Knockdown by RNA interference (RNAi) could be performed in order to perform functional evaluation of these miRNAs. In addition, mimic/transient transfection assays could be performed for those miRNAs whose expression is lost in holoclone samples. Data created by the next-generation sequencing analysis could be exploited to design the optimal RNAi and miRNA mimics for transfection assays.

8.3.4 Future Work: Quantification of Stem-associated Proteins in a Clinical Cohort

The fundamental aim of this study was to devise an *in vitro* prostate cancer stem cell model in order to explore the biology of cancer stem cells and identify distinct and most importantly unambiguous markers of 'stemness', therefore a natural extension of this work would be the analysis of the identified stem-associated genes (such as *EGR1* or *KITLG*) within a clinical cohort. As previously mentioned, the PCRC cohort represents a well-defined set of treatment-naive radical prostatectomy specimens, which will be monitored carefully in the coming years. Hence, the immunohistochemical analysis of proteomic targets such as EGR1 or TXNIP in PCRC tissue microarrays is warranted. Potentially, these proteins could represent potent markers of the prostate cancer stem cell population, whose quantification within primary tumour specimens may correlate with clinical features and disease progression.

8.3.5 Future Work: Analysis of Small RNA Species

To date, the involvement of the non-coding genome in the perpetuation of human malignancy has been investigated primarily in the context of microRNA dysregulation. However, the participation of other non-coding RNAs in human disorders is slowly being recognised. Indeed, miRNAs represent only a fraction of all the small non-coding RNA species encoded within the human genome which includes; PIWI-interacting RNAs (piRNAs, 36-31 bp), transcription initiation RNAs (tiRNAs, 17-18 bp), transcriptional start site associated RNAs (TSSa RNAs, 20-90 bp), small nucleolar RNAs (snoRNAs, 60-300 bp), promoter associated small RNAs (PASRs, 22 – 200 bp) and promoter upstream transcripts (PROMPTs, < 200 bp) (Esteller, 2011). This study has performed a next-generation sequencing analysis of all small non-coding RNA species up to 50 bp in size; therefore we have captured expression information on an abundance of small RNA elements.

PIWI-interacting RNAs (piRNAs) are so-called due to their interaction with the PIWI subfamily of Argonaute proteins. These PIWI proteins have been implicated in the maintenance of genome stability in germline cells (Aravin *et al.,* 2007). Generally, piRNAs

are not expressed in adult tissues; however piRNAs and piRNA-like transcripts have been associated with multiple tumour entities (Lu *et al.,* 2010; Park *et al.,* 2010; Yan *et al.,* 2011). The mechanisms underpinning the putative pathogenic effects of piRNAs and PIWI proteins are largely unknown. However, it is noteworthy that PIWI proteins have been implicated in the self-renewal of normal stem cells. Furthermore, the *piwil2* gene (of the PIWI/AGO family) is believed to regulate precancerous stem cells, which retain the propensity for malignant differentiation (Sharma *et al.,* 2001; Chen *et al.,* 2007). The observation that piRNAs are aberrantly expressed in cancer infers that this class of small non-coding RNAs may represent viable drug targets.

The association which is emerging between piRNAs and cancer stem cell characteristics indicates the necessity to perform a comprehensive bioinformatic analysis of the RNA-seq data generated by this study for piRNA species. As the non-coding RNA field expands and the functional roles of these small regulatory elements are clarified, piRNA repertoires of prostate holoclones will be put into context. Potentially, this work could establish a unique piRNA signature of prostate cancer stem cells, which, in theory, could be harnessed to achieve the fundamental goal of cancer stem cell eradication.

8.3.6 Future Work: Long ncRNAs

As previously discussed (7.3.6), characterisation of long non-coding RNAs has been arduous owing to the difficulties associated with the annotation of these transcripts. Despite extensive indications of functionality, the low sequence conservation of long non-coding RNAs has strengthened the assertion that they are non-functional. While experimental evidence negates this concept, lack of sequence conservation ensures that ncRNA function cannot simply be inferred by structure or sequence, as with miRNAs. At present, the functions attributed to long non-coding RNAs include; telomere biology, subcellular structural organisation and high-order chromosomal dynamics (Mercer *et al.,* 2009). This study has identified the differential expression of a number of novel intergenic RNAs. To confirm these transcript annotations, *in vitro* validation by RT-PCR and qPCR could be performed in PC-3 and DU145 parental cells. In order to explore these transcripts more closely, 5' and 3' rapid amplification of cDNA ends (RACE) could be performed. This analysis could identify the precise transcriptomic components of these loci and provide further insight into their function in the cancer stem cell phenotype. To explore the functional role of these lincRNAs in prostate cancer, knockdown experiments by short-interfering RNAs in PC-3 and DU145 cells could precipitate physiological responses from which, a putative function could be deduced. Gene expression profiling of knockdown samples may also reveal transcriptional targets of these ncRNAs.

To our knowledge, this is the first study to comprehensively analyse the transcriptome of putative prostate cancer stem cells. As such, RNA-seq data generated by this study represents an invaluable cache of transcriptomic data, which can be probed and computationally analysed in a multitude of ways. We have identified the downregulation of previously annotated lincRNAs in holoclone samples; however the RNA-seq data most likely contains additional novel unannotated lncRNA transcripts. Moreover, analysis of samples in isolation based on cell line origin could reveal differential lncRNA expression patterns. In this regard, this data contributes to a growing body of literature describing the role of non-coding RNAs in cancer stem cell biology. The expansion of this field may reveal the functional significance of lncRNAs identified through this study.

8.3.7 Future Work: Circular RNAs

Circular RNAs (circRNA) represent a recently (re)discovered transcriptomic phenomenon. These RNA molecules are postulated to be a consequence of back-splice events, which generate covalently bonded (at the 3'- and 5'-end) closed continuous RNA loops (Bachmayr-Heyda *et al.,* 2015). However, they were initially misinterpreted as splicing errors when they were first observed over 20 years ago (Nigro *et al.,* 1991). Circular RNA species possess no known function and as a result have become an area of intense study in recent years. It has been postulated that these long non-coding circular molecules act as 'miRNA sponges', which suppress the inhibitory activity of miRNAs, ultimately derepressing the miRNA target gene (Hansen *et al.,* 2013). While circular RNAs comprising exonic sequences have been described in a small number of genes, no association has been suggested between these novel RNA molecules and cancer stem cells. However, a recent study has proposed a correlation between circRNA abundance and proliferation within several human tumour tissue types (Bachmayr-Heyda *et al.,* 2015). Therefore, bioinformatic analysis of the RNA-seq data generated by this study to predict the presence of circRNAs utilising the pipeline described by Memczak *et al.,* (2013) may shed light on this relatively infantile area of research and provide further insight into the complex multi-layer regulatory circuits of prostate cancer stem cells.

8.4 Overall Conclusions

In this thesis, we have performed an expression analysis of a comprehensive Irish cohort of radical prostatectomy specimens in an attempt to define the pathological alterations associated with aggressive prostate carcinoma. In the coming years, it will become apparent whether the molecular signature unique to putatively 'aggressive' prostate tumours has the potential to portend clinical outcomes.

With regards to cancer stem cells, there remains a significant degree of contention surrounding the explicit ontogeny and phenotype of this cellular subset. As previously mentioned, increasing evidence is suggesting that the concept of heterogeneity and plasticity in the context of tumour progression cannot be defined by a fixed population of uniform 'cancer stem cells'. However it remains inarguable that plasticity is an omnipresent feature of human malignancy. Thus, the term 'cancer stem cell' should be viewed as a functional definition, which infers that cancer stem cells should be defined by their ability to self-renew and generate 'histocopy' tumours *in vivo*. Herein, we sought to characterise the molecular repertoires, which define the functional plasticity of prostate cancer stem cells through the generation of an efficacious *in vitro* model of 'stemness'. We have demonstrated that established prostate carcinoma cell lines can derive the functional and phenotypic plasticity native to somatic epithelial stem cells. These 'stem cell-containing' holoclones exhibit stem-associated gene expression patterns and efficaciously recapitulate prostate carcinoma within the murine host microenvironment. Furthermore, *in vivo* assays demonstrated the innate ability of these stem-like cells to initiate metastasis. A characteristic confirmed by the inherent downregulation of E-cadherin in holoclone-derived tumours. To address the scarcity of literature surrounding the molecular regulation of prostate cancer stem cells and to identify the putative genetic aberrations which define intrinsic plasticity, we performed an exhaustive analysis of the protein-coding and non-coding transcriptome of prostate cancer holoclones. This analysis has generated a complex illustration of the regulatory circuitry, which may define the prostate cancer stem cell phenotype.

To our knowledge, the PCRC study is the first of its kind to attempt a multi-modal characterisation of prostate cancer. In addition, no study has reported the comprehensive transcriptomic sequencing of prostate cancer holoclones. Thus, we have described the involvement of genes and miRNAs whose role in the stem phenotype has not previously been perceived. It is hoped that the research described within this thesis will contribute to the growing body of knowledge concerning disease progression and the involvement of prostate cancer stem cells. However, unravelling the complex, hierarchical, regulatory networks of cancer stem cells will rely upon further investigation.

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Appendices

Please see attached CD for Appendices 1 & 2