Investigating the Properties of Cancer Stem Cells and Epithelial to Mesenchymal Transition in Human Prostate Cancer

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<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
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<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating tumour cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothicyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epithelial growth factor receptor 2</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidise</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>miRNA</td>
<td>Microribonucleic acid</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Natural killer cell activating receptor</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
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<td>PCSC</td>
<td>Prostate cancer stem cell</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PEG</td>
<td>Primary epithelial growth media</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>SC</td>
<td>Stem cell</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TIC</td>
<td>Tumour initiating cell</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>WB</td>
<td>Western blotting</td>
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Abstract

In recent years, the cancer stem cell hypothesis has emerged as a compelling but controversial model of cancer progression. Contrary to the clonal evolution model, the cancer stem cell hypothesis postulates that, akin to normal tissues, tumours are hierarchical and only a rare subpopulation of cells, so-called “cancer stem cells” (CSCs), possess the unique biological properties required for tumourigenesis. In addition to tumour initiation, cancer stem cells are held solely accountable for tumour differentiation, tumour maintenance, tumour spread and tumour relapse following therapy. Of late, there has been much evidence to suggest that cancer cells reactivate the latent embryonic programme, epithelial to mesenchymal transition (EMT), in order to acquire the invasive and migratory properties necessary for the successful completion of the invasion-metastasis cascade. Intriguingly, the EMT programme was recently implicated in the generation of cells with the properties of stem cells in a breast cancer model, therefore, it is evident that multiple populations of CSCs may exist within a given tumour. Since metastasis is accountable for the vast majority of cancer-associated mortalities and CSCs are implicated in therapy failure and subsequent cancer relapse, it is apparent that epithelial to mesenchymal transition and cancer stem cells are of utmost clinical relevance. Consequently, this study sought to investigate the properties of CSCs and EMT in human prostate cancer. Using the method previously reported in the literature, this work failed to identify and isolate putative prostate CSCs. However, this study successfully identified the OPCT-1 cell line as a suitable model for the investigation of EMT and subsequently derived and selected five phenotypically distinct clones that provided a model to determine a possible correlation between EMT and the generation of CSCs in human prostate cancer.

The present work revealed that EMT of prostate cancer cells does not always generate cells with the properties of stem cells. Furthermore, this study demonstrated evidence for the existence of multiple cancer stem cell-like populations in human prostate cancer. In addition, evidence of EMT in primary cultures and clinical specimens from prostate cancer patients was shown and evidence to suggest that tumours contribute to their own stromal microenvironment via EMT was also provided. The foundations laid by the completion of this project provide considerable scope for future studies which may reveal valuable insights into the fundamental mechanisms involved in prostate cancer progression.
Chapter 1:

Introduction

1.1 Cancer: A Brief Overview

The word cancer is broadly used to describe a collection of over 200 diverse diseases which share a number of fundamental traits. It is essentially a disease process that is characterised by uncontrolled cellular proliferation and invasion of surrounding tissues which arise as a result of dysregulated cellular growth and cause a disruption of tissue homeostasis. Cancer can arise in almost any tissue of the body and some tissues may even yield multiple types. Several years of cancer research have contributed to an improved understanding of this complex group of diseases which, in turn, has led to rapid advances in cancer treatment. However, further efforts are undoubtedly required in order to discover uncharacterised mechanisms behind cancer initiation and its prevention.

1.1.1 Cancer: Facts and Figures

Cancer is accountable for a significant number of morbidities and mortalities worldwide; hence it represents a major health concern across the globe. In 2008, there were an estimated 12.7 million new cases of cancer worldwide and in 2010, there were 157,275 reported cancer deaths in the UK, which is an increase from the 156,723 deaths recorded in 2008 (Cancer Research UK, 2012). In 2010 cancer was by far the leading cause of death with 28% of all UK deaths resulting from cancer (Figure 1.1).

In 2010, almost half (47%) of all cancer deaths in the UK arose from cancers of the lung, bowel, breast and prostate (Figure 1.2). Although cancer continues to represent a considerable cause of death worldwide, due to advances in cancer detection and treatment over the years, mortality rates from cancer fell by a fifth (20%) between 1979 and 2008 (Cancer Research UK, 2010).
**Figure 1.1:** The 10 leading causes of death in the UK in 2010 (Cancer Research UK, 2012)

**Figure 1.2:** The 20 most common causes of death from cancer in the UK in 2010 (Cancer Research UK, 2012)
Cancer can affect people of all ages although more than three quarters of cancer deaths (77%) in 2010 occurred in people aged 65 and over. Therefore, the death rates from cancer rise with increasing age, with cancer being the leading cause of death in all age groups with the exception of 15 to 25 year olds. (Figure 1.3).

Some tissues are more susceptible to developing cancer than others and there is a notable difference in the pattern of the incidence and mortality of cancers between men and women (Figures 1.4 and 1.5) (King, 2000, Cancer Research UK, 2012).

Almost any site in the body can be subject to malignant transformation. The vast majority of cancers arise in epithelial cells and are known as carcinomas, cancers arising from glandular tissue (such as the prostate) are known as adenocarcinomas. Cancers which develop in cells of mesodermal origin, such as muscle, bone and fat cells, are known as sarcomas and cancers of the nervous system include neuroblastomas, retinoblastomas and astrocytomas. Not all cancers form solid tumours (abnormal growths) for example haematological cancers, known as leukaemias, are characterised by an abnormal increase of blood cells which circulate in the bloodstream (King, 2000).

The etiology of any given malignancy is complex, involving the interaction of a mixture of genetic, lifestyle and environmental factors. Therefore, cancer can be caused by both external and internal factors (discussed in detail in section 1.1.2) which may work synergistically to initiate or promote carcinogenesis up to several years after exposure (American Cancer Society, 2010).

![Figure 1.3: Age related incidence and deaths from cancer in men and women the UK in 2010 (Cancer Research UK, 2012)](image-url)
**Figure 1.4:** Cancer mortalities of UK men in 2010 (Cancer Research UK, 2012).

**Figure 1.5:** Cancer mortalities of UK women in 2010 (Cancer Research UK, 2012).
1.1.2 Carcinogenesis: A Multifactoral, Multistep Process

Carcinogenesis is the multistep process by which normal cells are transformed into cancer cells and occurs as a result of an accumulation of genetic abnormalities which affect vital regulatory pathways (King, 2000). The cells of the body are normally maintained under stringent developmental control hence, in order to sustain homeostasis, cells are required to differentiate, proliferate, migrate and apoptose under strict instruction from other cells in their vicinity. These tight controls ensure that each tissue size and architecture is appropriate to the requirements of the body (Weinberg, 1997). Cancer arises when cells become non-responsive to their developmental controls and proliferate excessively, which, in the case of solid malignancies, consequently results in the formation of tumours. Benign tumours, such as warts and adenomas are non-invasive and often remain encapsulated in connective tissue. These rarely pose a threat to life unless they form in enclosed spaces and compress vital structures or secrete high levels of hormones for example (Cancer Research UK, 2012). However, adenomas may progress to malignancy and give rise to adenocarcinomas (Talmadge and Fidler, 2010). Unlike benign tumours, malignant tumours are often fatal as they lack contact inhibition and grow in an invasive manner into surrounding tissues which may be vital for the survival of the organism as a whole. Moreover, they can also shed cells which are capable of spreading, via the bloodstream or lymphatic system, to other sites of the body where they can colonise and invade in a process known as metastasis.

Tumours are believed to derive from a single cell in a multi-step process which usually develops over many years and consists of different phases following a trend rather like that of Darwinian evolution; a process which depends on random mutations acquired over time to give rise to selective advantages to individuals (Vogelstein and Kinzler, 1993). Random mutations in genes controlling proliferation or apoptosis are strongly implicated in carcinogenesis and the mutations in individual genes which confer evasion of normal homeostatic controls are those which are detected in successful cancer cells (Bertram, 2001).

The majority of mutations that result in cancer are non-hereditary and arise spontaneously as a consequence of DNA damage which alters the function of vital genes (Bertram, 2001). Studies on colon carcinoma demonstrated that a successful human cancer cell requires mutations in at least five genes (Cho and Vogelstein, 1992; Cahill et al., 1999). Genomic
instabilities including point mutations (leading to amino acid substitutions), mutations resulting in truncated proteins or proteins with a scrambled sequence, chromosomal imbalance or instability (resulting in amplification), loss of a gene or fusion with another gene (as a consequence of chromosomal breakage and rearrangement), over-expression or inappropriate expression of a particular gene and epigenetic modifications to DNA (which may result in gene silencing) contribute towards the induction of malignant transformation of cells (Shar, 2001). The advent of modern sequencing technologies has enhanced our understanding of mutagenesis and revealed that malignant transformation is an extremely complex process requiring extensive genetic damage in the form of 1,000 to 100,000 mutations, depending on the individual and tumour type (Stratton et al., 2009). These mutations collectively confer malignancy by contributing to the autonomous growth of cancer cells in the host organism (Bertram, 2001).

1.1.2.1 Induction of DNA Damage

DNA damage resulting in malignant transformation most frequently arises as a result of exposure to physical, or chemical carcinogens which are encountered throughout life both internally and externally (Lodish et al., 2000). However, spontaneous DNA damage is also thought to give rise to cancers with no obvious cause (Bertram, 2001).

1.1.2.1.1 Spontaneous DNA damage

Studies using *Saccharomyces cerevisiae* as a model organism to investigate spontaneous DNA damage have revealed that deficiencies with nucleotide excision repair and base excision repair lead to elevated levels of unrepaired, spontaneous oxidative DNA damage producing genetically unstable cells reminiscent of cancer cells (Evert et al., 2004). In addition to deficiencies in DNA repair mechanisms, spontaneous DNA damage may arise as a result of the inherent instability of the DNA molecule, or oxidative damage from by-products of metabolism (Lodish et al., 2000; Bertram, 2001).

1.1.2.1.2 Environmental carcinogens

There is an abundance of frequently occurring factors with the ability to modify the physical structure of genes. As such, arguably the most common group of carcinogens are those readily encountered in the environment.
1.1.2.1.2.1 Chemical carcinogens

Carcinogenic chemicals include simple compounds, such as arsenic, and more complex compounds, such as the fungal chemical aflatoxin, which can be encountered as a result of ingestion, inhalation and direct skin exposure (King, 2000). As several compounds are capable of inflicting DNA damage, the list of chemical carcinogens is ever increasing. Perhaps the most renowned example of a chemical carcinogen is cigarette smoke, containing chemicals such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (BaP), which have been proven to induce lung cancer (Hecht, 1994).

1.1.2.1.2.2 Physical carcinogens

As with chemical carcinogens, physical carcinogens, in the form of radiation, are ubiquitous in the environment. Ionising radiation is prevalent from natural sources, such as the sun, industrial sources, such as nuclear fall-out, medical sources, such as x-rays and therapeutic radiotherapy, domestic sources, such as cathode ray tubes, and even in human bodies as a result of potassium-40 ingestion (Little, 2003). The carcinogenesis of electromagnetic fields remains a controversial topic, with some studies implicating a carcinogenic role (Alhbom et al., 2001) while others report on their therapeutic applications (Barbault et al., 2009). Skin cancer is an example of a malignancy which can be induced by artificial or solar ultra-violet radiation (Ananthaswamy, 2001).

1.1.2.2 Epigenetic Aberrations

While genetic abnormalities may account for some of the changes which lead to the progressive conversion of normal cells, many of the alterations in gene expression observed in cancer arise as a result of epigenetic modifications (Esteller, 2011). Epigenetic mechanisms include chromatin remodelling, DNA methylation, histone modification nucleosome remodelling, and RNA interference (Ducasse and Brown, 2006). Such mechanisms may be induced by hormones such as oestradiol, which plays an instrumental role in the initiation and progression of breast cancer (Mann et al., 2011).

Aberrant global levels of histone modification and incorrect methylation of gene promoter regions may lead to the activation of genes which promote malignant behaviour and/or repression of genes involved in protective mechanisms such as DNA damage repair and activation of apoptosis in response to irreparable insult (Santos-Reboucas et al., 2007) (See 1.1.2.4 Oncogenes and Tumour Suppressor Genes). Therefore, misregulation of
epigenetic mechanisms works synergistically with genetic mutations to drive the progression of neoplastic disease (Ducasse and Brown, 2006).

Unlike genetic mutations, epigenetic modifications are reversible. As such, small molecule inhibitors of the epigenetic enzymes are currently in development with DNA methyltransferase (DNMT) inhibitors and histone deacetylase inhibitors already approved modalities for the treatment of haematological cancers (Copeland et al., 2010).

1.1.2.3 Infection-induced Carcinogenesis

Induction of DNA damage in proliferating epithelial cells, as a result of repeated exposure to reactive oxygen species generated by inflammatory cells, is known to contribute to oncogenesis (Coussens and Werb, 2001). As such, cancer frequently arises in different tissues and organs as a consequence of chronic inflammatory disease. (Schaeffer and Werner, 2008). Accordingly, approximately 18% of all cancers worldwide are attributed to chronic infections with microorganisms including bacteria, viruses and parasitic worms (Thun et al., 2009). The most common malignancies induced by microorganisms include cervical, stomach and liver cancers, which are caused by, human papiloma virus (HPV), the bacterium Helicobacter pylori (H.pylori), and Hepatitis C virus (HCV), respectively (Thun et al., 2009).

Aside from causing chronic inflammation, RNA and DNA tumour viruses have been shown to contribute significantly to carcinogenesis by directly interfering with a variety of different host functions and are estimated to be the etiological agents of approximately 17% of human cancers (Parkin, 2006). Retroviruses transform cells via provision of cancer-causing “oncogenes” which play a role in mitogenic signalling and growth control whereas DNA viruses have been shown to transform cells via suppression of cellular repressor proteins known as “tumour suppressors” (Butel, 2000; King, 2000).

1.1.2.4 Oncogenes and Tumour Suppressor Genes

Normal cell growth and division is tightly controlled and regulated by stimulatory and inhibitory cell signalling pathways which convey growth-controlling messages from the outer surface to the nucleus of the cell. Within the nucleus “the cell cycle clock”, the molecular machinery which collects the messages, determines whether the cell should divide. Any mutations which hinder the genes involved in inhibitory pathways (known as tumour suppressor genes) may lead to excessive cellular proliferation (cancer).
Conversely, cancer may arise as a result of mutations which over-activate the genes involved in the stimulatory pathways (proto-oncogenes) (Weinberg, 1997).

The two gene classes, oncogenes and tumour suppressor genes play major roles in tumourigenesis. In a healthy cell, these genes are responsible for the maintenance of the cell cycle. However, when mutated they drive uncontrolled, excessive multiplication.

1.1.2.4.1 Proto-oncogenes and Oncogenes

Proto-oncogenes are genes expressed by normal, healthy cells which contribute primarily to cell signalling pathways involved in cell cycle progression, proliferation, angiogenesis and response to growth factors (King, 2000). As result of mutation, structural rearrangement or gene copy number gain, proto-oncogenes become altered or activated to form oncogenes, thereby resulting in disruption of their normal function which is exploited to promote tumourigenesis (Soh et al., 2009). Oncogenes have a dominant gain of function. Hence, cell growth becomes dysregulated when a mutation in a proto-oncogene renders a critical growth stimulatory pathway constitutively active when it should be silent. The growth-promoting properties of oncogenes are due to either a larger production or increased activity or sensitivity to protein products which endorse uncontrolled cellular proliferation in cancer (Weinberg, 1997). In the case of viral-induced oncogenesis, oncogenic retroviruses insert their oncogenes into the host cell’s DNA, thereby triggering uncontrollable cell division (Thorat et al., 2012).

Oncogenes are one of the most influential groups of genes in cancer and contribute significantly towards tumourigenesis. The first oncogene to be discovered in human cancer cells was the Ras oncogene, it is the most widely activated oncogene in all human cancers and was originally discovered in rat sarcomas (Bertram, 2001).

1.1.2.4.2 TumourSuppressorGenes

Overstimulation of growth-promoting machinery alone is not sufficient do drive malignancy as cells must also be capable of escaping and ignoring growth inhibitory signals (King, 2000). Tumour suppressor genes are of significant importance in human carcinogenesis and play an “anti-oncogenic” role by controlling cell growth, cell survival and DNA repair. Contrary to oncogenes, a recessive loss of tumour suppressor genes is associated with cancer development. Inactivation of tumour suppressors enables transformed cells to survive and continue to multiply thus favouring the progression of
malignancy. Therefore, inactivation of tumour suppressor genes is a common feature of cancer cells. p53 is a tumour suppressor gene which plays a critical role in conserving genetic stability and preventing genomic mutations via various mechanisms. Also referred to as “the guardian of the genome”, p53 can activate DNA repair, initiate growth arrest and induce apoptosis in response to stress (Weinberg, 1997). The key role of p53 in cancer prevention is emphasised by the fact that this gene is defective in over 70% of human cancers and almost 100% of individuals with hereditary mutations in p53, a condition known as Li Fraumani syndrome, suffer with cancer (Bertram, 2001).

1.1.2.5 Summarising the Pathway to Malignancy

Figure 1.6 demonstrates the steps involved in carcinogenesis. The process begins with a rare initiating event which causes genetic damage in a cell. Such an event may be induced by exogenous factors such as tobacco smoke, viruses and ionising radiation, or endogenous factors such as metabolic free radical generation or hormonal changes. A single (initial) event is not sufficient for tumourigenesis and progression to cancer requires promotional agents, such as hormones which stimulate mitosis and thereby promote further mutation (King, 2000). Mutations in DNA repair mechanisms and inhibition of apoptosis and replicative senescence enable malignant progression. Tumour formation results from uncontrolled cellular proliferation of immortal, self-renewing cancer cells which demonstrate the “hallmarks of cancer”.
**Figure 1.6:** Overview of pathways to malignancy. Demonstrating the stepwise acquisition of malignant traits via mutation and the inhibitory processes a tumour must overcome to survive and thrive, demonstrating the hallmarks of cancer. (Adapted from Bertram, 2001).
1.1.3 Defining the Features of Cancer

In January 2000, Douglas Hanahan and Robert Weinberg published a review “The Hallmarks of Cancer” which surmised six defining features common to all cancer types and in 2011 they revisited their concepts and introduced two additional, emerging hallmarks of cancer (Figure 1.7).

Figure 1.7: The Hallmarks of cancer. (Adapted from Hanahan and Weinberg, 2000;2011).
1.1.3.1 Self-Sufficiency in Growth Signals

As previously explained, normal (healthy) cells are tightly regulated and maintained via signalling and adhesion molecules from adjacent cells. In the absence of such molecules, normal cells are unable to move from an inactive, quiescent state into a proliferate state. However, as a result of oncogenic transformation, tumour cells are able to proliferate independently by producing and responding to their own growth signals in an autocrine manner. Acquired growth signal autonomy can be achieved at three levels; extracellular, via excessive production of growth signalling molecules, transcellular, via over-expression or structural changes of/to growth factor receptors, or intracellular, via alterations in downstream cytoplasmic signalling molecules (Hanahan and Weinberg, 2000).

1.1.3.2 Insensitivity to Growth-Inhibitory Signals

Unless normal cells are required to proliferate, they are maintained in a quiescent state by tumour suppressor genes which issue anti-proliferative signals to maintain tissue homeostasis. Cells with sustained DNA damage apoptose under the control of tumour suppressor genes. However, inactivating mutations in both alleles of tumour suppressor genes render them unable to regulate the cell cycle and promote apoptosis in response to irreparable DNA damage. As a result, transformed cells continue to proliferate in the absence of DNA repair mechanisms and become increasingly genomically unstable. An accumulation of mutations in proto-oncogenes and tumour suppressor genes consequently allows cells to progress towards a malignant cell phenotype.

Tumour suppressor proteins can be inactivated directly by protein binding or phosphorylation. Alternatively, mutation of tumour suppressor genes can result in an altered protein product or complete loss of product. The p53 gene is the most frequently altered tumour suppressor gene in human cancers and there have been over 2000 p53-inactivating mutations identified (King, 2000).

1.1.3.3 Inexhaustible Self-Renewal (replicative) Capacity

Normal cells have a finite lifespan hence, once they have progressed through their maximum number of population doublings, they lose their ability to divide in a process known as senescence. It is a natural phenomenon otherwise known as “aging” and is attributed to the progressive erosion of telomeres, the ends of chromosomes, during successive cycles of replication. Unlike normal cells, tumour cells appear to exhibit
limitless replicative potential, hence are immortal. The immortality of tumour cells has been attributed to their ability to maintain their telomeres by either upregulating the expression of the telomerase enzyme, or by activating a mechanism which maintains telomere length through recombination. Both mechanisms maintain telomeres at a length which enables unlimited replication of successive cells thus defying the natural aging process (Hanahan and Weinberg, 2000). Interestingly, the role of telomerase in conferring inexhaustible replicative capacity can be demonstrated in vitro through transformation of normal cells with a plasmid construct expressing telomerase.

In recent years, an increasing body of evidence has developed to support “the cancer stem cell theory”, a theory that implicates stem cells or stem-like cells in tumourigenesis. In addition to multilineage differentiation potential, stem cells are known to possess a robust capacity for clonal self-renewal. Recent studies have provided evidence to suggest that cancer arises as a result of malignant transformation of tissue stem cells (Reya et al., 2001; Richardson et al., 2004; Collins et al., 2005; Bjerkvig et al., 2005; Ailles and Weissman, 2007; Bapat, 2007; Huang et al., 2006; Lawson and Witte, 2007; Neuzil et al., 2007; Gao, 2008). The pathways involved in the regulation of normal stem cell self-renewal have been found to be deregulated in cancer stem cells thus rendering them capable of uncontrolled, infinite expansion which results in tumour formation (Al-Hajj and Clarke, 2004). Furthermore, stem cells have been shown to inherently express higher levels of telomerase than somatic cells. Therefore, the self-renewal property of cancer cells could be attributed to the fact that they derive from transformed tissue stem cells. Cancer stem cells will be discussed further in Section 1.5.

1.1.3.4 Reprogramming of Energy Metabolism

In order to fuel the rapid cell growth and proliferation exhibited by cancer, cancer cells must adjust their energy metabolism accordingly. For this purpose, cancer cells have long been known to reprogram their glucose metabolism, hence, energy production, by limiting their energy metabolism to glycolysis, even in the presence of oxygen. This phenomenon was first observed by Otto Warburg, thus, is referred to as “the Warburg effect” in addition to “aerobic glycolysis”. This metabolic switch is accompanied by an upregulation of glucose transporters, which substantially increase the import of glucose into the cytoplasm thereby partially compensating for the ~18-fold lower efficiency of ATP production afforded by glycolysis in comparison to mitochondrial oxidative phosphorylation.
(Hanahan and Weinberg, 2011). The hypoxic cancer cell is dependent on glycolysis for energy metabolism. Therefore, glycolysis is upregulated by the transcription factors HIF-1\(\alpha\) and HIF-2\(\alpha\) which increase in response to hypoxia. Interestingly, by increasing the levels of HIF-1\(\alpha\) and HIF-2\(\alpha\), the Ras oncoprotein can also upregulate glycolysis (Hanahan and Weinberg, 2011).

Warburg-like metabolism also appears to be present in several rapidly proliferating embryonic tissues and stem cells. This will be discussed further in Section 1.5.5.5.3.

### 1.1.3.5 Evasion of Immune Destruction

The immune system is comprised of multiple cells, proteins and other factors which collectively work to seek and destroy/neutralise invading pathogens, infected cells, toxins and cancer cells. However, cancer cells are armed with several mechanisms for evading immune destruction therefore, are able to thrive in spite of the immune system.

The key cells of the immune system for tumour surveillance are T-cells, which form part of the adaptive immune response (Igney and Krammer, 2002). Tumours can escape from the main line of defence (T-cells) by downregulating the expression of classical HLA class I antigens, effectively rendering them invisible to these immune cells but susceptible to attack by NK cells (Rees et al., 1988; Ahmad et al., 2004).

The expression of surrogate HLA molecules can be employed by cancer cells to resist-NK-mediated lysis. HLA-G is a non-classical HLA class I molecule involved in immunomodulation. Its expression is generally restricted to cytotrophoblasts, embryonic tissues, erythroid precursors, corneal and thymic epithelial cells and stem cells (Lozano et al., 2003). However, aberrant HLA-G expression by tumour cells has also been observed in several human malignancies and has been proposed to play multiple roles in immune evasion, in either its soluble or membrane-bound forms (Rouas-Freiss et al., 2005). Soluble HLA-G has been detected in body fluids of cancer patients such as serum and effusion samples and is believed to be secreted by the capable tumour cells in order to inactivate the local immune response, even for tumour cells without cell-surface HLA-G expression (Sheu and Smith, 2010). This molecule is known to play multiple immunosuppressive functions exerted by inhibition of several immune effector cells. Direct inactivation of immune effectors such as T-cells, B cells, monocytes (macrophages), myeloid dendritic cells and NK cells is achieved via direct interaction between HLA-G and leukocyte
immunoglobulin-like receptors LILRB1 and LILRB2 (Cosman et al., 1997). In addition, HLA-G directly interacts with the NK inhibitory receptor KIR2DL4 resulting in inhibition of NK-cell-mediated cytolytic activity (Clements et al., 2007). Other studies have revealed that immune tolerance, mediated by HLA-G can be achieved via induction of regulatory T-cells (Le Rond et al., 2006).

Therefore, it is evident that the aberrant expression of this immunosuppressive molecule can greatly assist the immune evasion of both the tumour cells which express membrane-bound HLA-G and those in the vicinity of the cells which secrete this molecule. These examples represent only a few of the vast mechanisms of HLA-G mediated immunosuppression. For a more detailed insight into the role of HLA-G in cancer, the reader is referred to reviews by Sheu and Shih, 2010 and Rouas-Freiss et al., 2005.

Cytokines are also known to play an important role in immune evasion and tumours have been shown to secrete high levels of specific cytokines, which reduce the activity of the local immune cells. Transforming growth factor beta (TGF-β) is an example of an immunosuppressive cytokine which affects proliferation, activation and differentiation of cells of innate and adaptive immunity, hence, inhibits the anti-tumour immune response. This cytokine may be secreted directly from tumour cells or by activated tumour-associated stromal cells. In addition, several other immunosuppressive factors have been shown to be expressed by tumour cells, these include VEGF, which inhibits the differentiation of progenitors to dendritic cells, prostaglandins, interleukin 10 (IL-10) macrophage-colony stimulating factor and soluble tumour gangliosides (Igney and Krammer, 2002). These examples demonstrate that cancer cells are equipped with an array of defensive mechanisms which render them resistant to immune attack. It is important to acknowledge that the examples provided here outline only a few of the multiple strategies employed by cancer cells to evade immune destruction. For a more detailed overview, the reader is referred to the review articles by Ahmad et al., 2004 and Igney and Krammer, 2002.

1.1.3.6 Resistance to Apoptosis (Cell Death)

Normal tissue growth is maintained by a balance between cellular proliferation, differentiation and death. There are two types of cell death, necrosis and apoptosis. Necrosis is the passive process by which a cell breaks down in response to a poor nutrient
supply or acute cellular injury which leads to a loss of membrane integrity. Apoptosis, also known as “cell suicide”, is the active process of programmed cell death and requires the synthesis of macro molecules. Apoptosis is a normal process involved in tissue remodelling and tissue growth or atrophy is determined by the rate of proliferation versus the rate of cell death. The apoptotic pathway can be induced in response to a number of different external events which trigger regulatory proteins to initiate the pathway in response to stress. Such events include; DNA damage, accumulation of toxins, withdrawal of growth cytokines, viral infection, nutrient deprivation and hypoxia. The induction of apoptosis results in the activation of a family of cysteine proteases (caspases) which promote the release of cytochrome C from mitochondria into the cytoplasm thus activating additional caspases which cleave cellular proteins and bring about cellular degradation without inducing an inflammatory response (King, 2000; Bertram, 2001).

Mounting evidence indicates that an acquired ability to resist apoptosis is a characteristic that is common to most, if not all, forms of cancer. Cancer cells can acquire the ability to resist apoptosis through various different strategies. One example of which is the inactivation of p53 as a result of mutation to both alleles, or production of proteins which inhibit its transcriptional activity (e.g. HPV E7) (Massimi and Banks, 1997). p53 plays a critical role in the induction of apoptosis in response to DNA damage by upregulating the expression of pro-apoptotic Bax, a potent stimulator of cytochrome C release. In the absence of p53, cells with sustained DNA damage are able to escape apoptosis and continue to proliferate in the absence of DNA repair mechanisms which subsequently results in increasing genomic instability (Hanahan and Weinberg, 2000).

1.1.3.7 Sustained Angiogenesis

Angiogenesis (neovascularisation) is the vital physiological process by which new blood vessels form from pre-existing vessels. In a normal tissue environment the formation of new blood vessels is a natural process which is tightly maintained.

In the absence of new capillaries, tumours cannot grow beyond 1-2 mm in diameter as further growth must be supported with a fresh blood supply (Talmadage and Fidler, 2010). Therefore, in order to expand, tumour cells promote angiogenesis by producing angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF-1 and FGF-2). Furthermore, tumours are capable of suppressing anti-angiogenic factors such as thrombospondin (Hanahan and Weinberg, 2000). Interestingly,
these angiogenic factors appear to be closely associated with tumour suppressor genes and onco-genes. For example, where the tumour suppressor gene p53 positively regulates the angiogenic inhibitor thrombospondin, the ras onco-gene is associated with an increased secretion of VEGF (Bertram, 2001). In addition to these mechanisms, the cancer stem cell component has been shown to be directly capable of vessel wall formation via differentiation into endothelial cells (Ricci-Vitiani, et al., 2010; Wang et al., 2010). The contribution of cancer stem cells to angiogenesis will be discussed in more detail in Section 1.5.5.5.6.

1.1.3.8 Tissue Invasion and Metastasis

Providing that transformed cells possess all of the characteristics previously discussed, they can multiply excessively to form a clinically detectable mass of cells referred to as a primary tumour. Unlike normal cells, which exhibit contact inhibition, the cells of a primary tumour are able to invade and disrupt surrounding tissues (Voet and Voet, 1995). However, the disruption to homeostasis caused by the invading cells of a primary tumour is rarely fatal and the vast majority of deaths due to cancer are as a result of metastasis. Being responsible for over 90% of cancer-associated mortality, metastasis is of paramount clinical significance (Brabletz, 2012). Metastasis is an extremely complicated and arguably inefficient process, involving several distinct steps which must be executed by successful carcinoma cells. The so-called “invasion-metastasis cascade” describes the series of events required for this enormous undertaking (Fidler, 2003). In order to complete the invasion-metastasis cascade, malignant cells must escape from the primary tumour site, penetrate the local stroma, enter the local vascular or lymphatic vessels, in a process termed intravasation, aggregate with platelets, interact and adhere to distant endothelia, escape from the circulation via extravasation, recolonise in a foreign environment and expand to form secondary tumours, all the while avoiding immune destruction and surviving in multiple microenvironments (Thompson and Newgreen, 2005). Given the complexity of the process and the incredible challenge with which the carcinoma cells are faced throughout their dissemination from the primary site to their arrival and colonisation at the secondary sites, metastasis remains a controversial and poorly understood phenomenon, with more than one mechanism proposed.

It is highly probable that carcinoma cells are not equally endowed with the properties required for metastasis, as evidenced by the fact that the presence of tumour cells in the
circulation does not predict metastasis. In fact, \textit{in vivo} studies using radiolabelled cancer cells injected intravenously revealed that most are efficiently eliminated (Talmadge and Fiddler, 2010). The notion of heterogeneity among cancer cells is further supported by the inefficiency of metastasis, which rarely exceeds 0.01% despite the abundance of tumour cells shed daily into the blood stream of cancer patients (up to a million per gram of tumour) (Talmadge and Fidler, 2010).

Emerging concepts in cancer biology are offering new insights into the previously unexplainable phenomena observed in cancer metastasis. The cancer stem cell hypothesis and epithelial to mesenchymal transition have both been largely implicated in metastasis and will be discussed in more detail in sections 1.5 and 1.6.
1.2 Prostate Cancer

Prostate cancer is a major cause of morbidity and mortality in men, particularly in the developed world (Drewa and Styczynski, 2008). It is the third most common cancer in men worldwide and is most prevalent in European and North American men (Gronberg, 2003). Prostate cancer accounts for a quarter of all new cancer cases diagnosed in men in the UK, hence it is the most common male malignancy in the UK (Cancer Research UK, 2012). In 2010, 10,721 men died of prostate cancer in the UK (Cancer Research UK, 2012). Prostate cancer occurs when tumours present in the prostate gland. The prostate, which is located between the bladder and the rectum, composes part of the male reproductive system and is involved in the production and storage of seminal fluid. Prostate cancer can present with an absence of symptoms however, common symptoms include changes in urinary or sexual functions. Urinary symptoms may present as a result of prostatic swelling which can affect the flow of urine and, since the prostate is involved in production of seminal fluid, damage to this gland can affect sexual function (Caroll et al., 2005).

In 2008 around 913,000 men were diagnosed with prostate cancer worldwide. With more than 40,000 cases of prostate cancer being diagnosed annually in the UK, the incidence of prostate cancer has almost tripled over the last 30 years. However, much of the increase has been attributed to better detection through widespread use of the prostate specific antigen (PSA) test (Cancer Research UK, 2012).

Prostate cancer is predominantly a disease of the aged male with less than 0.1% of cases occurring in males under the age of fifty and more than three quarters of cases occurring in men over the age of sixty five (Cancer Research UK, 2012). Therefore, with increasing age there is an increased risk of prostate cancer (Figure 1.8). Although the strongest risk factor for prostate cancer is age, other factors are known to play a role. For example, there is an increased risk of prostate cancer in first-degree relatives of those diagnosed with prostate cancer, particularly if they were diagnosed under the age of sixty. West African and black Caribbean men are known to have two to three times more risk of developing prostate cancer than white men whereas Asian men are at a lower risk of developing prostate cancer than any other group.
Although genetic factors undoubtedly account for some of the associated risk, lifestyle factors are known to play a large role since Asian men living in the UK have an increased risk of developing prostate cancer compared with Asian men living in Asia (Cancer Research UK, 2010; Cancer Research UK, 2012).

Due largely to earlier detection, as a result of increased PSA testing in the UK, more than three quarters (>81%) of men diagnosed with prostate cancer now survive beyond five years. This is compared with less than a third (<33%) of men in the 1970s (Cancer Research UK, 2012). Prostate cancer death rates have decreased by approximately 20% since the early 1990s when they peaked. That said however, after lung cancer, prostate cancer is still the second most common cause of cancer death in UK men and each year an estimated 10,150 men in the UK die from this disease (Cancer Research UK, 2010).

Localised prostate cancer is curable by surgery and radiation therapy and the most widely used therapy against advanced prostate cancer is androgen ablation which almost always produces objective and clinical responses (Miki et al., 2007; Miki and Rhim, 2008). However, the majority of patients relapse with ablation-resistant prostate cancer which develops into metastatic disease (Miki and Rhim, 2008). Therefore, the management of disease spread is a difficult task (Drewa and Styczynski, 2008). There is currently no
treatment to cure progressive hormone-refractory metastatic prostate cancer (Miki and Rhim, 2008).

Resistance to hormone ablation therapy and progression to hormone-refractory prostate cancer may be governed by a population of cancer cells which exhibit stem-like properties. Indeed, there has been much evidence implicating epithelial prostate stem cells as targets for malignant transformation in prostate cancer and this has been the topic of several well-written reviews and research articles (Collins et al., 2005; Maitland et al., 2006; Lawson and Witte, 2007; Miki et al., 2007; Signoretti and Loda, 2007; Kasper, 2008; Maitland and Collins, 2008; Miki and Rhim, 2008). The role of cancer stem cells in prostate cancer initiation and progression has sparked enormous interest and, as such, prostate cancer stem cells are currently the subject of intense investigation.
1.3 Stem Cells

1.3.1 Stem Cells: A Brief History

In recent years, the topic of stem cells has roused much excitement, controversy and promise. Through various isolation and transplantation techniques, stem cells are hoped to hold the key to the treatment of several incurable diseases. During the 1950’s, a series of experiments by radiation biologists elucidated that the reintroduction of whole bone marrow cells to lethally irradiated mice could save them from an inevitable death (Houghton et al., 2007). Further work, conducted by Till and McCulloch in the 1960’s, found that the restoration of a functional haematopoietic system was accompanied by colony formation in the spleen. Furthermore, they found that the cells of these colonies were capable of forming more colonies when injected into a second mouse (Till and McCulloch, 1961). These influential studies led to the first strict definition of so called “colony forming cells”, which we now refer to as “stem cells”.

1.3.2 Stem Cell Characteristics

Stem cells are the founders and sustainers of most, if not all, tissues and organs in the body (Szilvassy et al., 2003). The ultimate, totipotent, stem cell is the fertilised oocyte which gives rise to a complex organism composed of approximately 200 types of specialised somatic cells (Wobus, 2001). During embryonic development, the fertilised egg initially generates progeny that retain totipotency. As the cells continue to divide and determination occurs the cells begin to lose their ability to differentiate into multiple cell types and instead begin to gain the specialised functions necessary to generate mature organs (Sell, 2004a). The primary germinal layers, the ectoderm, endoderm and mesoderm are pluripotent and give rise to all tissues in the body.

The role of stem cells is not restricted to tissue formation during embryogenesis as they are also known to reside in adult tissues and play an essential role in tissue repair, reconstitution and maintenance (Suzuki and Nakauchi, 2002). Adult stem cells are believed to generate cells committed to specific tissues and organs and are thought to play a particular role in the maintenance and repair of such tissues throughout life (Sanders Jr et al., 2006). Depending on the complexity of the tissues they form and maintain, stem cells vary in differentiation potential (potency). Haematopoietic stem cells, for example, are
pluripotent, whereas prostate stem cells are an example of unipotent stem cells (Sell, 2004b).

One of the main hallmarks of stem cells is their ability to “self-renew” almost indefinitely without senescence. Depending on which signals they receive, stem cells are capable of proliferating by symmetrical division, giving rise to two identical daughter (stem) cells, and asymmetrically, giving rise to an identical, quiescent daughter (stem) cell and an active differentiating (progenitor/transit amplifying) daughter cell (Verfaillie, 2002). Such properties are unique to stem cells and therefore, distinguish them from progenitor or precursor cells, which are unable to self-renew indefinitely (Friel et al., 2005). The generation of mortal progenitor/transit amplifying cells to take on the role of differentiation enables the stem cells to remain in a relatively dormant state thereby protecting the genome of the stem cell while delegating the genetically dangerous task of repeat-replication to a dispensable cell (Houghton et al., 2007).

Stem cells are generally considered to constitute a minute population amongst a majority of differentiated, specialised cells. As such, one of the challenges of stem cell research is the identification of specific markers which distinguish stem cells from their more differentiated progeny. Furthermore, stem cells have been shown to reside in the so-called “stem cell niche”, the specific microenvironment which plays a critical role in stem cell homeostasis and differentiation of its progeny (Miller et al., 2005). Therefore, in order to maintain the potency of stem cells in culture, or to induce differentiation to generate stem-cell progeny, it is necessary to recreate the correct microenvironment, as far as possible, using specific growth factors which either promote symmetrical division or induce differentiation, respectively.

The functional identification of a tissue stem cell involves demonstrating that a single stem cell is capable of reconstituting a given tissue in vivo. Although, to date, this remains to be demonstrated for the majority of candidate stem cells (Houghton et al., 2007). Nonetheless, this property has generated much interest in the scientific world due to hopes that stem cells may be exploited for a number of different clinical applications such as organ transplantation and repair of damaged tissues as a result of serious injury.

Since totipotent stem cells derive naturally from embryonic tissue, despite the great potential and the expectation that it will revolutionise biomedical research, human
embryonic stem cell research has been the topic of much ethical controversy which has hindered the applications of human embryonic stem cells (Takahashi et al., 2007).

1.4 Induced Pluripotent Stem Cells

The induction of pluripotency in somatic cells is a new field which holds great promise for stem cell research. Since human embryonic stem cells may have a multitude of applications, ranging from disease treatment to drug screening, in 2006, Yamanaka and colleagues developed a method to circumvent ethical issues by inducing pluripotency in somatic mouse cells by direct reprogramming. This feat was achieved by exploiting a retrovirus to transf ect the four transcription factors, Oct3/4, Sox2, c-Myc and Klf4 into murine fibroblasts to induce reprogramming into pluripotent stem cells which exhibited the morphology, growth properties and genetic signature of embryonic stem cells (Takahashi and Yamanaka, 2006).

Following the success of induced pluripotency in mouse fibroblasts, this group went on to generate induced pluripotent stem (iPS) cells from human somatic cells, which they found to be comparable to human embryonic stem cells in terms of morphology, gene expression, proliferation, surface antigen expression, epigenetic status of pluripotent cell-specific genes, telomerase activity and teratoma formation (Takahashi et al., 2007).

The concept of induced pluripotency has sparked intense interest and excitement in recent years, particularly because it may provide the means to generate patient-specific stem cells (Takahashi et al., 2007).
1.5 Cancer Stem Cells

1.5.1 Cancer Stem Cells: An Overview

Cancer is a class of diseases known to affect all vertebrate species and has been studied extensively for decades. Although many of the mechanisms involved in carcinogenesis have been elucidated, several questions, including the cell of origin of cancer, remain unanswered (Houghton et al., 2007). The stochastic and clonal evolution models are among several models used to describe cancer development. The general consensus is that cancer may arise in any cell as a result of many genetic mutations in oncogenes and tumour suppressor genes which consequently result in uncontrolled cell growth. Most models claim that every cell within a tumour is capable of reconstituting new tumours (Gao, 2007). However, the cancer stem cell hypothesis challenges previous opinion and states that:

“Tumours are hierarchical like normal tissues and only the rare subpopulation of cells at the pinnacle of that hierarchy have the unique biological properties necessary for tumour initiation.” (Lawson and Witte, 2007).

Where the stochastic theory implies that cancer cells have equal malignancy, the cancer stem cell hypothesis opposes this by suggesting that only a rare subpopulation of cells have the biological properties required to initiate and sustain tumour growth.

Cancer stem cells and their potential role in tumourigenesis have been the topic of much debate in recent years as they may help to elucidate some of the unknown phenomena in cancer research including cancer relapse and metastasis.

1.5.2 The History of the Cancer Stem Cell Hypothesis

Although cancer stem cells have only recently become the subjects of intense investigation, the stem cell theory of cancer dates back over 100 years, when parallels were drawn between the histology of certain tumours (such as teratocarcinomas) and the developing foetus (Houghton et al., 2007). However, the discovery of oncogenes and tumour suppressor genes in the 1970s shifted the focus away from the stem cell theory as Nowell formulated the clonal evolution concept (Nowell, 1976; Clevers, 2011).

Recent, advances in stem cell biology have led to the identification of adult tissue stem cells in various organs which, in turn, has enabled research into such populations as
potential candidates for malignant transformation. In 1997, Bonnet and Dick prompted huge interest in the stem cell theory of cancer when they implicated adult (haematopoietic) stem cells in tumourigenesis. In their study Bonnet and Dick demonstrated that a single rare leukaemia cell (present at 1 in 250,000 cells), with the cell surface marker expression pattern of haematopoietic stem cells (CD34+/CD38-), was capable of inducing leukaemia that was phenotypically identical to the parent tumour when transplanted into non-obese diabetic/severe combined immunodeficient (NOD/SICD) mice (Bonnet and Dick, 1997). In demonstrating tumourigenesis by these rare cells, Bonnet and Dick provided compelling evidence for the role of stem cells in the initiation of cancer. Consequently, others sought to identify tumour-initiating cells (aka cancer stem cells) from heterogeneous populations of cells in solid malignancies. In 2003, Al-Hajj et al., were the first to successfully identify and prospectively isolate cells with in vivo tumour-forming ability from a solid human tumour (breast). Since then, putative cancer stem cells have been identified and isolated from several other solid malignancies including prostate, pancreatic, brain, colon, head and neck, lung, liver, ovarian and skin cancers (Singh et al., 2003; Collins et al., 2005; Szotek et al., 2006; Li et al., 2007; Ricci-Vitiani et al., 2007; Zhao et al., 2008; Visvader and Lindeman, 2008).

1.5.3 What is a Cancer Stem Cell?

The cancer stem cell is hypothesised to be the original cell of a tumour which is held solely responsible for tumourigenesis, tumour differentiation, tumour maintenance, tumour spread (metastasis) and tumour relapse following therapy (Moltzahn et al., 2008). Analogous to normal stem cells, cancer stem cells have been shown to demonstrate a marked capacity for proliferation, self-renewal and differentiation and are believed to constitute a very rare population of cells amongst a majority of non-tumourigenic tumour cells. Furthermore, like normal stem cells, CSC’s are known to divide both symmetrically, giving rise to more CSC’s, and asymmetrically to give rise to progenitor-like cells which then go on to differentiate (Table 1.1). Unlike the bulk of the tumour, which consists of rapidly proliferating cells in addition to post-mitotic, differentiated cells, CSCs have the capacity to self-renew therefore, contribute to the long-term sustenance of the tumour (Clevers, 2011).

Aside from their self-renewal and differentiation capabilities, cancer stem cells have been shown to share many other properties with adult stem cells. For example, in accordance
with the numbers of normal stem cells reported in healthy tissue, cancer stem cells are frequently reported to comprise only 0.1-2% of the mass of a tumour (Ailles and Weissman, 2007). Furthermore, cancer stem cells can spawn phenotypically diverse progeny with variable proliferative potential (Zhao et al., 2008). Akin to normal stem cells, CSCs are reported to be highly resistant to drugs and toxins, through the expression of drug efflux pumps and high aldehyde dehydrogenase activity, and are resistant to radiotherapy and apoptosis, through a variety of mechanisms including active DNA-repair (Testa et al., 2007, Baumann et al., 2008; Iannolo et al., 2008; Johanness et al., 2008; Baumann et al., 2009; Fulda and Pervaiz, 2010; Koch et al., 2010; Wang et al., 2010; Zhang et al., 2010) (See Section 1.6.3.8). Considering these facts, the proposed properties of cancer stem cells may explain why disease relapses occur in many cancers, since it is believed that current cancer therapies only eliminate the bulk of the tumour, leaving the (resistant) cancer stem cells behind to reinitiate tumour growth (Neuzil et al., 2007).

Figure 1.9 summarises the cancer stem cell hypothesis and reveals that CSC’s could potentially arise from mutated adult tissue stem cells (SC), mutated progenitor cell/transit amplifying cells or differentiated cells which acquire mutations conferring de novo self-renewal and differentiation capabilities. Although the origin of the cancer stem cell has not yet been elucidated for every tumour type, there is evidence to support all three mechanisms and perhaps CSC’s can arise by any one of the three mechanisms proposed.
**Figure 1.9:** The cancer stem cell hypothesis. Cancer stem cells may arise from the malignant transformation of adult tissue stem cells, progenitor/transit amplifying cells or differentiated cells which acquire stem-like properties. Conventional therapies are unable to target, resistant, cancer stem cells which reside and subsequently repopulate the tumour. Combination therapy targeting the rapidly diving bulk of the tumour and the quiescent cancer stem cells is required in order to achieve complete tumour eradication (Dunning et al., 2011).
Table 1.1 Demonstrating the properties of normal stem cells and cancer stem cells and the surface markers of each from different tissues (adapted from Zhao et al., 2008; Dunning et al., 2011).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Cancer</th>
<th>CSC Markers</th>
<th>Normal SC Markers</th>
<th>References</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CD34^+CD38^+Thy1^-Lin^-</td>
<td>CD34^+CD38^+Thy1^-Lin^-</td>
<td>Baum et al., 1992; Lapidot et al., 1994</td>
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1.5.4 What is the Origin of Cancer Stem Cells?

Although the cancer stem cell theory draws parallels between normal tissue stem cells and tumour-initiating cells, thus far, several studies have failed to address whether or not cancer arises due to the transformation of normal stem cells and rather suggests that, irrespective of the cell of origin, cancers are comprised of a heterogeneous population of cells which may be organised in a hierarchical manner much like normal tissues (Shackleton et al., 2009). Hence, like normal tissues, which are maintained by a small population of self-renewing stem cells, tumours are thought to contain a stem-like component which drives proliferation and the origin of such a component is controversial, with more than one theory for the origin of cancer stem cells having been proposed.

1.5.4.1 Evidence to Suggest CSCs Derive from Adult Stem Cells

There is much evidence to support the notion that tumours frequently originate from the malignant transformation of adult tissue stem cells. Not only are adult tissue stem cells the only cells believed to live long enough to accumulate sufficient genetic mutations and epigenetic modifications for malignant transformation, CSCs from several neoplasms have been shown to share many common markers with the normal tissue stem cells (Table 1.1). For example, when one considers the average epidermal cell turnover, which is 6 weeks for humans, it is evident that only a long-lived stem cell could be subjected to the extensive neoplastic changes required to induce malignancy (Bergtresser and Taylor, 1977; Miller et al., 2005). The stem cell origin of corneal epithelial carcinomas is compellingly evidenced by the fact that the only carcinomas associated with this tissue occur in the peripheral corneal region, the site of corneal epithelial stem cells (Waring et al., 1984; Schermer et al., 1986; Miller et al., 2005). Haematopoietic stem cells are believed to be the source of malignant transformation for chronic myeloid leukaemia and acute lymphoblastic leukaemias which demonstrate the Philadelphia chromosome. The Philadelphia chromosome occurs as a result of a translocation between chromosome 9 and 22 and gives rise to the Oncogenic fusion gene BCR-ABL. As this cytogenetic abnormality can be found in 95% of CML patients in myeloid, erythroid, megakaryocytic and B lymphoid cells, the original translocation is believed to occur in a haematopoietic stem cell (Sawyers, 1999; Cobaleda et al., 2000; Miller et al., 2005). Perhaps the most compelling evidence, implicating stem cells in tumour initiation, comes from studies of childhood leukaemia in
identical twins, revealing a common clonal origin in some instances, thus indicating that the initiation occurred prenatally (Greaves, 2003).

These examples collectively provide evidence to suggest that, in some cancers, adult tissue stem cells are the site of malignant transformation hence, are the source of the tumour-initiating cancer stem cells.

1.5.4.2 Evidence to Suggest CSCs Derive from Progenitor/Transit Amplifying cells

Although there is a strong case implicating stem cells as the original tumour-initiating cells, studies have also provided evidence to suggest that, in some cancers, cancer stem cells arise from the transformation of more committed, progenitor cells. Given the relatively shorter life-span of progenitor cells, it is most likely that, for these cases, the initial genetic insults arise in stem cells but the final transforming events take place downstream, in early progenitor/transit-amplifying cells (Miller, 2005; Argyle and Blacking 2008). Through studies on human and murine tumours carrying the BRCA1 mutation, several groups have implicated luminal progenitor cells as the likely cell of origin for both luminal and basal-like breast carcinomas (Castano et al., 2012). Studies on brain tumour development revealed that neural progenitor cells, which reverted to a stem-like form following the acquisition of mutations conferring self-renewal abilities, were the likely candidates of malignant transformation (Zhao et al., 2008).

1.5.4.3 Evidence to Suggest CSCs Derive from Differentiated cells

There is also evidence to suggest that in certain contexts, paracrine interactions and epigenetic mechanisms may bestow stem-like properties on differentiated cells, a phenomenon referred to as dedifferentiation (Castano et al., 2012). In addition to paracrine and epigenetic causes, it is plausible that activating mutations in otherwise silenced stem-cell genes such as OCT3/4, SOX2, and C-MYC may confer stem-like properties in differentiated cells. This is supported by the fact that it is possible to reprogram differentiated cells to induce pluripotency via transfection with the aforementioned genes (Takahashi et al., 2007; Yamanaka, 2007).

In 2011, Chaffer et al., demonstrated that both normal and cancer stem-like cells could be generated de novo from more differentiated cells spontaneously in culture, thereby demonstrating plasticity in differentiated cells (Chaffer et al., 2011).
In recent years, great interest has developed in the latent embryonic programme, shown to be reactivated in some cancer cells, known as epithelial to mesenchymal transition (EMT) (See section 1.6). Although a link between this phenomenon and metastasis had been demonstrated previously, in 2008 Mani et al., released a groundbreaking paper which connected two fields of cancer research by establishing a link between EMT and the generation of cells with the properties of stem cells, thereby demonstrating that cancer-stem-like cells could be generated via transdifferentiation of cancer cells (See section 1.6) (Mani et al., 2008).

1.5.4.4 Other Sources of Cancer Stem Cells

It is important to note that the three origins proposed are based on the most reported hypotheses of cancer stem cell development and are likely to be over-simplified. In future, new insights into phenomena such as cell fusion and transdifferentiation will undoubtedly introduce new hypotheses addressing other plausible mechanisms of cancer stem cell development (Moltzahn et al., 2008).

1.5.5 Evidence and Rationale for the Fxistence of CSCs

Despite the fact that the cancer stem cell theory provides an explanation for many of the unexplained phenomena observed in cancer, such as tumour initiation, tumour heterogeneity and relapse following therapy, it is not accepted by all (Hill, 2006). However, despite criticism there is a large body of evidence to support the existence of a stem-like component in several malignancies.

1.5.5.1 Cancer Stem Cells Share Several Properties With Normal Adult Stem Cells

As previously described, putative cancer stem cells isolated from both cell lines and primary cultures have been shown to exhibit several properties common to normal adult stem cells (Table 1.1).

1.5.5.2 Tumours are Heterogeneous

Several studies have demonstrated heterogeneity among the cells present in cell lines, primary cultures, patient tumours and xenograft tumours.

Heterogeneity in the behaviour of tumour cells is evidenced by the fact that only 1 in 1,000 to 1 in 5,000 neuroblastoma, lung cancer and ovarian cancer cells have been demonstrated
to form colonies in soft agar, thereby revealing that only a small proportion of cells from these malignancies possess colony forming abilities (Argyle and Blacking, 2008). In addition, sphere forming assays on cancer cell populations revealed that only a small population of cells, seeded at clonal density, were capable of anchorage independent growth, a property considered unique to stem cells (Dontu et al., 2003; Mani et al., 2008; Kong et al., 2010). Furthermore, depletion of putative cancer stem cells from the bulk of the tumour cells often leads to a decrease in in vivo tumourigenicity (Al-Hajj et al., 2003; Wei et al., 2007), whereas, as few as 100 putative cancer stem cells have been shown to be capable of tumour initiation in mice (compared to tens of thousands of cells depleted of cancer stem cells which failed to form tumours) (Al-Hajj et al., 2003).

These studies demonstrated the existence of small populations of cells with enhanced clonogenicity, sphere-forming capability and tumourigenicity among a majority of non-clonogenic, non-sphere-forming and non-tumourigenic cells, findings which are consistent with the cancer stem cell hypothesis.

1.5.5.3 Pathways That Regulate Normal Stem Cells are Implicated in Oncogenesis

The balance between self-renewal and differentiation of stem cells is tightly maintained by regulatory signalling pathways. Critical proteins involved in the regulation of proliferation, differentiation and self-renewal of healthy stem cells include Sonic Hedgehog, Wnt, Notch and Bmi-1 (Moltzahn et al., 2008). Disruptions in these pathways, leading to up or down-regulation, have been shown to contribute to tumourigenesis in several cancers (Miller et al., 2005). In fact, many of the genes involved in Hedgehog signalling are known oncogenes which can also function as tumour suppressors, high levels of β-catenin, an integral component of the canonical Wnt pathway, have been observed in most common human malignancies, and, depending on the cell type, Notch can function as an oncogene or a tumour suppressor gene (Zhao et al., 2008).

These findings provide evidence for the stem cell origin of cancer, as mutations in inherent stem cell pathways, leading to the activation of oncogenes or inhibition of tumour suppressors, have been shown to contribute to tumourigenesis.
1.5.5.4 Cancers Demonstrate Expression of Embryonic Stem Cell Markers

Tumour development has long been acknowledged to resemble abnormal embryogenesis (Pierce and Speers, 1988). In recent years, several groups have identified the expression of embryonic stem (ES) cell markers in several human cancers (Jeter et al., 2009; Schoenhals et al., 2009; Guo et al., 2011). Such markers included Oct4, Sox2, Nanog, Klf4 and c-Myc, which have all been shown to induce pluripotency in somatic cells (Takahasi et al., 2007; Theunissen et al., 2011). Therefore, these genes are known to play key roles in the maintenance of pluripotency and self-renewal in stem cells.

In rectal tumours, the expression of Oct4 and Sox2 were shown to correlate with poor disease free survival, thereby revealing a deleterious role of these transcription factors in cancer (Saigusa et al., 2009). In addition, Nanog has been shown to contribute to tumour development in prostate cancer patients via promotion of CSC characteristics and resistance to androgens (Jeter et al., 2011).

Therefore, not only have embryonic stem cell markers been identified in human tumours, their expression has also been demonstrated to correspond with tumour development and poor patient prognoses. Cancer stem cells are believed to be the real driving force behind tumour development and progression therefore, the correlative expression of ES cell markers in aggressive tumours is consistent with the cancer stem cell hypothesis.

1.5.5.5 Cancer Stem Cells Denote the Hallmarks of Cancer

Perhaps the most important aspect of cancer stem cells is that they confer the so-called “hallmarks of cancer”, first outlined by Hanahan and Weinberg in 2001 and revised in 2011 (See section 1.1.3)

1.5.5.5.1 Sustainability of Proliferative Signalling and Evasion of Growth Suppressors

As previously outlined, activation of oncogenes and inhibition of tumour suppressor genes is a long, multistep process which contributes greatly to the malignant transformation of cells and it has been argued that only stem cells live long enough to acquire enough mutations and epigenetic modifications to confer malignancy (Miller et al., 2005). Several studies have demonstrated the significance of stem cell signalling pathways in oncogenesis and revealed the oncogenic and/or tumour suppressor functions of key genes in these pathways (Moltzahn et al., 2008; Zhao et al., 2008). As such, it is feasible that an
accumulation of mutations and epigenetic modifications in adult tissue stem cells could lead to the malignant transformation of these cells and subsequent processes involved in tumourigenesis.

1.5.5.2 Demonstration of Replicative Immortality

The telomeres of most somatic cells shorten with each cell division and once the telomere shortens beyond the critical length, DNA damage pathways are activated, leading to cell death or replicative senescence (Hiyama and Hiyama, 2007). Telomerase is a complex of a reverse transcriptase protein which adds telomeric repeats onto chromosome ends to prevent the replication-dependent loss of telomere and subsequent cellular senescence in highly proliferative cells such as germline cells and embryonic stem cells (Hiyama and Hiyama, 2007). Therefore, telomerase activity enables prolonged cellular proliferation. To maintain tissue homeostasis and the balance between proliferation and cell death, with the exception of adult stem cells, telomerase expression is silenced in most adult somatic tissues. Telomerase activity in adult stem cells grants these cells the longest telomeres within a given tissue, thereby enabling them to undergo multiple cell divisions before they finally senesce (Rajaraman et al., 2006; Marion and Blasco, 2010). Although telomerase is usually expressed at low levels in stem cells, during periods of heightened proliferation it is known to be upregulated (Haik et al., 2000). Furthermore, telomerase activity is readily detected in most cancers (Aranois and Greider, 2005). It was previously believed that cancer cells reactivate telomerase in late stages of cancer progression, however, the malignant transformation of adult tissue stem cells which already demonstrate telomerase activity is, perhaps, a more feasible explanation.

Therefore, the expression of telomerase by cancer cells conceivably reflects one of the inherent properties of the cells from which they derive (stem cells).

1.5.5.3 Reprogramming of energy metabolism

Akin to cancer cells, stem cells have been shown to exhibit the “Warburg effect” whereby they maintain anaerobic metabolism, even in the presence of oxygen (aka aerobic glycolysis) (Varum et al., 2011). This process enables these cells to survive in hypoxic environments. In fact, it is believed that self-renewal and differentiation of stem cells are well maintained in hypoxia whereas proliferation dominates with increasing oxygen levels (Bjerkvig et al., 2009). The reprogramming of energy metabolism is yet another feature
shared by stem cells and cancer cells, and the PTEN/PI3K/Akt pathway, involved in
glycolysis and often activated in cancer, has been shown to be particularly up-regulated in
cancer stem-like cells (Kendall et al., 2008). This pathway is also critical for the self-
renewal of haematopoietic stem cells (Miyamoto et al., 2007). However, when activated in
cancer, the PI3K pathway confers a competitive growth advantage, metastatic competence
and, often, therapeutic resistance (Hennessy et al., 2005).

Cancer cells and stem cells, therefore, both demonstrate reprogramming of energy
metabolism thus, revealing another connection between cancer and stem cells.

1.5.5.5.4 Avoidance of Immune Destruction

Among tumour cells, cancer stem cells are particularly enhanced in their ability to evade
immune destruction. Immune recognition and consequent destruction of cancer stem cells
are actively disturbed by a number of processes including altered immunogenicity of
CSCs, production of CSC-derived regulatory molecules and direct interaction between
CSCs and immune cells (Qi et al., 2012). In addition, transdifferentiation of cancer cells to
mesenchymal stem-like cells via epithelial to mesenchymal transition (See Section 1.6)
may provide an additional mechanism of immune evasion.

Cancer stem cells have been shown to exhibit loss of MHC class I molecules and
selectively silence tumour associated antigen expression thereby resulting in resistance to
immune rejection. This has been demonstrated in ABCB5-positive malignant melanoma
initiating cells (MMICs) with absent/downregulated MHC class I molecules and in MMICs
which lacked expression of the tumour-associated antigen MART-1 (melanoma antigen
recognised by T-cells) (Qi et al., 2012). One study, investigating the immunogenicity of
putative astrocytoma and glioblastoma cancer stem cells (CD133+ cells), revealed that the
majority of CD133 expressing cells did not express detectable MHC class I or NK cell-
activating ligands. Therefore, not only would such cells prove resistant to adaptive and
innate immune surveillance, they would appear to be unsuitable targets for
immunotherapy. However, upregulation of MHC class I and NK cell ligands was
successfully achieved following treatment with interferon gamma hence the
immunogenicity of the cells was restored (Wu et al., 2007).

Expression of the immunosuppressive HLA-G molecule is generally restricted to a few cell
types including stem cells. However, HLA-G has also been detected in several human
malignancies and, via several molecular mechanisms, this molecule induces immunosuppression thereby granting a selective advantage to cancer cells (Sheu and Shih, 2010). Very recently, studies have begun to reveal the expression of HLA-G by putative cancer stem cells and metastatic bone marrow-infiltrating cells (Brown et al., 2012; Morandi et al., 2012). The expression of HLA-G in putative cancer stem cells may provide evidence to suggest that these cells arise from the malignant transformation of adult tissue stem cells, which inherently express HLA-G. Alternatively, HLA-G expression on metastatic stem-like cells may occur as a result of EMT which has been shown to generate cells with the properties of mesenchymal stem cells (Battula et al., 2010). This is feasible as mesenchymal stem cells have been shown to constitutively express HLA-G in addition to secreting other immunomodulatory molecules such as transforming growth factor beta (TGF-β) (Nasef et al., 2007; Qi et al., 2012).

The expression of HLA-G by cancer stem cells may explain why these cells are not successfully eradicated by the immune system and hence, why tumour growth is permitted. In addition to alterations in HLA status, cancer stem cells have been shown to suppress T-cell immunity via secretion of immunosuppressive cytokines including interleukin 4 (IL-4), interleukin 10 (IL-10) and TGF-β (Qi et al., 2012). High levels of IL-4 and its receptor have been directly detected in CD133-positive putative colon cancer stem cells whereas glioblastoma stem cells were shown to inhibit interferon gamma (IFN-γ) secretion whilst upregulating IL-10 secretion by peripheral blood mononuclear cells (PBMCs) in co-culture (Sabbagh et al., 2009; Qi et al., 2012). Transforming growth factor beta has been shown to directly target the function of cytotoxic T lymphocytes (CTL), having a negative effect on their anti-tumour capabilities (Thomas and Massague, 2005). Shipitsin and colleagues (2007) demonstrated that the TGF-β pathway was specifically activated in the cancer stem cell component of malignant breast tissue. In line with this finding, secreted morphogens of the TGF-β superfamily and their receptors were shown to be preferentially expressed by CD133-positive brain cancer stem cells and ABCB5-positive MMICs (Piccirillo et al., 2006; Schatton et al., 2008; Qi et al., 2012).

Collectively, these studies demonstrate the immunosuppressive role of putative cancer stem cells via a wide range of mechanisms, some of which are innate to normal stem cells.
1.5.5.5 Resistance to Apoptosis

As previously explained, akin to normal stem cells, cancer stem cells are capable of resisting apoptosis using a number of different mechanisms. Examples of these include the secretion of IL-4, which has been reported to inhibit apoptosis by enhancing the expression of anti-apoptotic proteins; PED, cFLIP and Bcl-XL in many cancer cell lines and increased expression of apoptosis-inhibiting genes such as Bcl-2, c-I, AP2, XIAP and NAIP by CD133-expressing putative glioblastoma stem cells (Qi et al., 2012; Conticello et al., 2004). Furthermore, putative glioblastoma stem cells were shown to down-regulate pro-apoptotic genes at the mRNA level (Liu et al., 2006).

In addition to inherent mechanisms, the induction of epithelial to mesenchymal transition by cancer cells has been shown to contribute to apoptotic resistance (Hollier et al., 2009) which may be due to the generation of mesenchymal stem-like cells. This is arguable because mesenchymal stem cells have been shown to be highly resistant to chemotherapy-induced apoptosis (Mueller et al., 2006).

Therefore, cancer stem cells demonstrate heightened resistance to apoptosis and, as such, are thought to contribute significantly to the failure of therapy and disease relapse (See Section 1.6.3.8)

1.5.5.6 Induction of Angiogenesis

Several studies have revealed cancer stem cells to be the most strongly angiogenic cells within a tumour (Ribatti, 2012). Indeed, blood vessel-forming cancer stem cells expressing markers of vascular endothelium have even been described (Ricci-Vitiani, et al., 2010; Wang et al., 2010). In addition, brain tumour stem cells have been shown to interact with brain tumour endothelial cells, which were shown to promote the expansion of brain CSCs and accelerate tumour growth (Calabrese et al., 2007). The differentiation of cancer stem cells to endothelial cells was achieved when Ricci-Vitiani et al., cultured glioblastoma stem-like cells in conditions favourable for the differentiation of stem cells into endothelial cells. This was subsequently validated in an in vivo murine model when injected glioblastoma stem cells generated large anaplastic tumour xenografts demonstrating vessel wall formation by human endothelial cells. This was also confirmed in studies by Wang et al., 2010.
In addition to being capable of transdifferentiating into endothelial cells, cancer stem cells have been shown to produce elevated levels of the pro-angiogenic vascular endothelial growth factor (VEGF), compared with the bulk of the tumour (Bao et al., 2006).

Therefore, cancer stem cells have been shown to contribute significantly to angiogenesis, even more so than their more differentiated counterparts.

1.5.5.7 Activation of Invasion and Metastasis

Epithelial to mesenchymal transition (EMT) is largely implicated in metastasis and will be discussed in more detail in Section 1.6.

In addition to the cancer stem-like cells generated by EMT, there is evidence to suggest the role of other stem-like populations in invasion and metastasis. In recent years, researchers have demonstrated cellular heterogeneity within the cancer stem cell compartment, with some CSCs demonstrating enhanced metastatic capabilities (Sampieri and Foddlle, 2012). For example, Hermann et al., 2007 revealed that CXCR4-positive pancreatic cancer cells within the CD133-positive CSC compartment demonstrated enhanced migratory capacity thus, were shown to possess high malignant and metastatic potential (Herman et al., 2007). Furthermore, in an in vivo model, only the CXCR4-positive/CD133-positive cells gave rise to liver metastases. Similarly, Pang et al., 2010 identified a subpopulation of CD26-expressing colon cancer stem cells. These cells were detected in both primary and metastatic tumours isolated from high-grade colorectal cancer patients however, only one third of the primary tumours contained a population of CD26-positive cells compared to 100% of the metastases examined (n=16). Furthermore, in vivo studies revealed that, although CD26-negative cells were tumourigenic, only CD26-positive cells gave rise to distant metastases (Pang et al., 2010). Perhaps the most compelling evidence demonstrating the role of CD26-positive cancer stem cells in metastasis was the fact that none of the patients without CD26-positive cells in their primary tumours developed metastases, whereas the majority of patients with CD26-positive cells did (Pang et al., 2010).

Further evidence implicating CSCs in metastasis was demonstrated by Charafe-Jauffret et al., (2009), who revealed that breast cancer cells expressing high levels of aldehyde dehydrogenase 1 (ALDH1), an enzyme involved in stem cell survival and early differentiation, were significantly more metastatic in an in vivo model. This finding was
confirmed in a similar study by Croker et al (2009). In another study, ALDH1 was shown to be a predictor of poor clinical outcome for breast cancer patients (Ginestier et al., 2007).

Collectively, these data provide compelling evidence for the critical role of cancer stem cells in metastasis and, through a variety of mechanisms, it is evident that cancer stem cells exhibit the hallmarks of cancer, stipulated by Hanahan and Weinberg (2000; 2011). It is however, apparent that tumours present with more than one stem-like cell population; the cells which may represent transformed stem/progenitor/dedifferentiated cells, and the mesenchymal stem-like cells generated by EMT, both of which appear to contribute significantly to tumourigenesis. The existence of more than one so called “cancer stem cell” population will be discussed in more detail in Section 1.6.3.7.

1.5.6 Identification, Isolation and Enrichment of CSCs

As cancer stem cells are reported to constitute a rare population of cells within a given tumour, it is essential to accurately identify these cells prior to isolation. Direct identification of CSC’s can be achieved by flow cytometry-based assays using characteristic surface or internal markers, DNA labelling and assessing the ability of CSCs to actively expel dyes, such as Hoechst 33342 using drug transporter proteins. In addition, fluorescence microscopy is frequently used to identify cells expressing putative cancer stem cell markers. However, these methods are not entirely robust since CSC markers may be common to other cell types and some progenitor cells are also known to possess active drug efflux mechanisms conferred by the ABC transporter ABCG2, which has been implicated in multiple drug resistance (Argyle and Blacking, 2008). In addition, DNA labelling is reliant on the quiescent nature of CSC’s which is common to normal tissue stem cells (Moltzahn et al., 2008). Therefore, due to the inability to distinguish between normal and cancer stem cells in many cases, none of the CSC identification methods are completely reliable.

Table 1.1 shows some of the most common cell surface markers used to identify and isolate cancer stem cells and normal stem cells. Perhaps the two most common cell-surface CSC markers, which have been used to identify putative cancer stem cells from several tissues, are CD44 and CD133, as such, antibodies to identify these markers and kits to isolate them from a mixed population of tumour cells are readily, commercially available. Putative cancer stem cells can be isolated based on the expression of cell
surface-proteins by cell sorting or alternatively, by magnetic-activated cell sorting (MACS), a technique which is argued to be gentler on the cells than cell sorting. However, since CSCs constitute a rare population of the tumour, an enrichment step may be required in order to generate enough CSCs to interrogate thoroughly. Enrichment steps may include those which exploit the unique properties of cancer stem cells to grow under certain conditions, such as sphere-forming assays, in which tumour cells are grown in non-adherent conditions to enrich for cells capable of anchorage independent growth, or the use of harsh treatments which only the resistant cells, including the CSC population, can survive. Such treatments include the use of chemotherapeutic agents and radiation since the ABC transporters and active DNA repair mechanisms permit the resistant CSCs to reside (Huang et al., 2007).

In order to satisfy the conditions of a cancer stem cell, several criteria must be tested, exploiting the specific growth characteristics unique to cancer stem cells. Numerous assays have been used to assess cancer “stemness”, the most common of which are colony forming assays (a), assessing cancer stem cell proliferation and differentiation capabilities, sphere-forming-based self-renewal assays (b), whereby spheres are grown and repeatedly dissociated and re-seeded in order to assess the ability of CSCs to self-renew indefinitely, and in vivo transplantation of enriched CSCs in immunocompromised mice (c), confirming their tumour-initiating capability and self-renewal capacity (Figure 1.10).

**Figure 1.10:** Common cancer stem cell assays a) colony forming assay, b) self-renewal assay, C) in vivo tumourigenesis assay (Adapted from Visvader and Lindeman, 2008)
Once putative cancer stem cells are isolated from the bulk of the tumour population, it is important to maintain them in defined serum-free conditions, with specific growth factors which promote self-renewal while inhibiting differentiation. There are several commercially available media and supplements for the maintenance of normal and cancer stem cells.
1.6 Epithelial to Mesenchymal Transition

1.6.1 Epithelial to mesenchymal Transition: An overview

Epithelial to mesenchymal transition (EMT) is a complex biological process which is activated from the beginning of life, during implantation and embryogenesis, throughout life, during organ remodelling and tissue repair in response to injury, and in pathological states such as organ fibrosis and cancer. The EMT programme enables polarised epithelial cells to undergo multiple biochemical changes, permitting them to assume a mesenchymal phenotype - which is entirely different from that of an epithelial cell (Kalluri, 2009).

1.6.2 Epithelial and Mesenchymal Tissues

The differences between epithelial and mesenchymal tissues are illustrated using a prostate gland as an example (Figure 1.11). Although epithelia may originate from any of the three germ layers, ectoderm, mesoderm or endoderm, all epithelial tissues share several cellular and molecular characteristics (Blanpain et al., 2007). Epithelial tissues line the cavities of the human body and comprise the main functional unit (parenchyma) of many glands, such as the prostate. These tissues perform several critical functions including protection, filtration, absorption, diffusion and secretion (Hollier et al., 2009). For example, the secretory luminal epithelial cells (Figure 1.11, blue cells) of the prostate secrete prostatic fluids containing prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) into the lumen. Differentiated epithelial cells possess a rich network of cytokeratin intermediate filaments, which can distinguish them from mesenchymal cells. Epithelial cells are well organised in continuous sheets of cuboidal or columnar cells connected laterally by intercellular adhesion complexes (Foroni et al., 2012). Such adhesion complexes, including tight junctions, adherens junctions, gap junctions and desmosomes, ensure tight connections between adjacent cells. The epithelial layer is polarised, with distinction between the two sides, one being defined as apical and the other as basal. Apical-basal polarity, localised distribution of adhesion molecules and polarised organisation of the actin cytoskeleton are maintained by the anchorage of the epithelial cells to the basement membrane in addition to the lateral cell-cell adhesive junctions. Such adhesive structures maintain the shape and position of the epithelial cells, thereby preventing their migration away from the epithelial layer and entry into the underlying stromal compartment (Hollier et al., 2009).
As is evident, in contrast to the epithelial compartment, the stromal compartment is comprised of loosely organised mesenchymal cells and fibroblasts which are not closely associated with the basement membrane (Figure 1.11). Unlike epithelial cells, these cells form only weak contacts with adjacent cells, display spindle-like morphology and exhibit front to back polarity (Hollier et al., 2009). In addition to morphological features, mesenchymal cells are commonly identified by their expression of the intermediate filament vimentin. Mesenchymal cells function to support the growth of the parenchyma via production and secretion of extracellular matrix proteins and growth factors. These cells are highly mobile and produce and secrete extracellular matrix degrading enzymes which facilitate their invasion and migration through surrounding tissues.

Figure 1.11: A functional prostate gland, illustrating the differences between the epithelial and mesenchymal (stromal) cells. (Adapted from Czyż et al., 2012).
1.6.3 The EMT Programme

The EMT programme involves a complex sequence of events which enable polarised epithelial cells, which are typically tightly bound to one another and associated with the basement membrane, to shed their epithelial traits and assume a mesenchymal cell phenotype (Figure 1.12). Thereby conferring increased motility, via disassembly of cell-cell contacts and acquired vimentin expression, enhanced invasiveness, via production of extracellular matrix degrading enzymes, increased production of extracellular matrix proteins, immunosuppressive function and increased resistance to apoptosis. (Kalluri and Weinberg, 2009; Kudo-Saito et al., 2009). This process is marked by a decrease in the expression of epithelial markers, such as cytokeratins, E-Cadherin and pakoglobin and a concomitant increase in the expression of mesenchymal-associated markers including vimentin, N-Cadherin, smooth muscle actin (SMA), fibronectin and matrix metalloproteinases (MMPS) (Hollier et al., 2009). A shift from cobblestone morphology, characteristic of epithelial cells, to spindle-like, fibroblastic morphology is also evident in monolayer culture (Figure 1.13), along with increased motility which can be observed using time-lapse imaging.

Several reversible molecular processes are engaged for the initiation and progression of EMT, a programme involving activation of numerous transcription factors, suppression and upregulation of specific microRNAs (miRNAs), drastic reorganisation of the cytoskeletal network, suppression and upregulation of specific cell-surface proteins and synthesis of extracellular matrix proteins and MMPs. The epigenetic nature of the activators of this programme in several contexts often renders it a transient and reversible process. Indeed, the reverse transition from a mesenchymal to an epithelial phenotype (MET) is also known to occur and, among other functions, is known to play a critical role in the generation of mesoderm-derived epithelial organs such as the kidneys (Thiery et al., 2009). Therefore, this cyclic process is characterised by high phenotypic plasticity and is predominantly activated and regulated by environmental factors and contextual signals (Figure1.12) (Brabletz, 2012).
**Figure 1.12:** Epithelial to mesenchymal transition (EMT) and the reverse process mesenchymal to epithelial transition (MET). This figure demonstrates that epithelial cells are highly plastic and that the transitions between epithelial and mesenchymal states are often transient therefore, cyclic. Key proteins characteristic of either epithelial or mesenchymal cells are listed, along with some of the significant events and the effectors which regulate EMT and MET. ECM – extracellular matrix, FGFR2 – fibroblast-growth-factor receptor 2, FSP1 – fibroblast-specific protein-1, MFs – microfilaments. (Adapted from Thiery and Sleeman, 2006).
Figure 1.13: Examples of epithelial and mesenchymal morphologies observed in clonally derived populations from the prostate cancer cell line OPCT-1 in 2D culture. A) Pure population of epithelial cells exhibiting typical cobblestone morphology B) Pure population of EMT-derived mesenchymal cells with characteristic spindle-like morphology C) Mixed population of epithelial and mesenchymal cells with mesenchymal cells surrounding the colonies of epithelial cells. Image magnification at x10.
Epithelial to mesenchymal transitions contribute to distinct biological processes where they vary in functional consequences. In some settings, EMT contributes to essential developmental and homeostatic processes however, in other contexts it contributes towards disease states and may have fatal consequences. Based on the biological and biomarker context in which they occur, epithelial to mesenchymal transitions are classified into three subtypes, Types 1-3 (Kalluri, 2009).

1.6.3.1 Type 1 EMT: Involved in Implantation, Embryogenesis & Organ Development

Several Type 1 epithelial to mesenchymal transitions are known to accompany and underlie embryonic morphogenesis. For example, following the earliest stages of embryogenesis, Type 1 EMTs play a role in embryonic implantation and initiation of placental formation. Subsequent formation of the three germ layers during gastrulation is also an EMT-dependent process, as the primitive ectodermal cells of the primitive streak initiate the EMT programme prior to ingression and migrate to eventually form the endoderm and mesoderm, thereby transforming the embryo from a single layer to three germ layers (Kalluri and Weinberg, 2009; Foroni et al., 2012). Indeed, the significant role of EMT in this process is emphasised by the fact that without EMT, gastrulation cannot occur, hence, embryonic development is halted at the blastula stage (Hollier et al., 2009). The EMT process is also key in the development of the neural crest, whereby cells of the neural plate gain a mesenchymal phenotype via EMT to enable their migration through the periphery where they differentiate into different cell types (Kalluri and Weinberg, 2009). In addition to these examples, Type 1 EMT has also been implicated in several other developmental processes which will not be discussed here.

1.6.3.2 Type 2 EMT: Involved in Tissue Regeneration and Organ Fibrosis

Type 2 EMTs are activated in the context of inflammation, in response to growth factors such as TGF-β, EGF and FGF2 secreted by inflammatory cells, and are associated with wound healing, tissue regeneration and organ fibrosis. In contrast to Type 1 and Type 3 EMTs, which generate mesenchymal cells, Type 2 EMTs generate fibroblasts from mature epithelial cells (Hollier et al., 2009; Zeisberg and Neilson, 2009).

1.6.3.2.1 Wound Healing and Tissue Regeneration

The activation of Type 2 EMT in response to epithelial tissue injury has been shown to facilitate wound healing and restore the original tissue architecture. This can be
demonstrated using *in vitro* models, whereby an artificial scratch is introduced to a confluent monolayer of epithelial cells which are then monitored during re-growth or by *in/ex vivo* wound healing assays conducted on mice. These assays reveal that epithelia assume a mesenchymal phenotype in order to migrate and close the artificial wound, *in vitro*, or in order to invade the surrounding tissue and migrate to the wound site to participate in re-epithelialisation, *in/ex vivo* (Hollier *et al*., 2009; Vuoriluoto *et al*., 2010).

The upregulated expression of the transcription factor Slug/Snail2, a direct repressor of E-Cadherin known to play an influential role in EMT, in cells bordering *in vitro* and *in/ex vivo* wounds provides further evidence for the role of EMT in tissue regeneration. Furthermore, ectopic expression of Slug has been shown to accelerate wound healing (Bolos *et al*., 2002; Savagner *et al*., 2005).

### 1.6.3.2.2 Organ Fibrosis

In addition to assisting essential physiological processes, epithelial to mesenchymal transitions may also contribute to pathological processes. Organ fibrosis, which occurs as a result of Type 2 EMT in response to inflammation and/or tissue damage, may interfere with organ function and may prove fatal. The significant contribution of Type 2 EMT in renal fibrosis is highlighted by the fact that over a third of the fibroblasts which accumulate in the interstitials of diseased kidneys originate from tubular epithelia at the site of injury (Kalluri and Neilson, 2003). In addition, there is evidence to suggest that endothelial cells undergo an analogous transition EndMT and contribute to diseases such as cardiac fibrosis (Kalluri and Weinberg, 2009).

Malignant transformation may occur as a severe complication of non-healing ulcers and the development of cancer in fibrotic tissue is also a common event (Schaefer and Werner, 2008). Many of the mechanisms which govern wound healing are known to regulate tumour progression (Hollier *et al*., 2009). Analogous observations made between the composition of healing wounds and tumour stroma led Dvorak, 1986, to propose that “tumors are wounds that do not heal”. This analogy is supported by the fact that, akin to healing wounds, cancer cells have been shown to activate epithelial to mesenchymal transitions.
1.6.3.3 Type 3 EMT: Involved in Tumour Progression and Metastasis

In recent years, Type 3 EMT has been proposed as the critical mechanism commandeered by epithelial cancer cells, resulting in the acquisition of malignant characteristics enabling invasion, migration, apoptotic resistance and immunosuppression, all of which are required for progression through the invasion-metastasis cascade. Furthermore, in 2008, Mani et al., linked EMT with the acquisition of stem-like properties thereby accounting for the characteristics required for the successful establishment of macrometastases (Mani et al., 2008).

Interestingly, evidence of Type 2 EMTs generating fibroblasts can be observed in the context of cancer. For example, fibroblasts adjacent to primary epithelial tumours have been shown to share some genetic mutations with the cancer cells, suggesting that they may have arisen as a result of Type 2 EMT prior to the full onset of tumourigenesis (Kurose et al., 2002). However, studies comparing mutations in cancer cells and cancer-associated fibroblasts (myofibroblasts) isolated from distant metastases failed to denote a cancer cell of origin for these fibroblasts, thereby suggesting that epithelial tumour cells are no longer a source of fibroblasts (Zeisberg and Neilson, 2009). It is worth noting that unlike Type 2 EMT, Type 3 EMT is not a mechanism for generating fibroblasts and is rather a means to acquire an invasive and migratory phenotype to enable successful completion of the invasion-metastasis cascade (Zeisberg and Neilson, 2009).

It is now widely accepted that as cancer progresses, some cells at the periphery of the primary tumour are capable of transitioning (via EMT) into a mesenchymal state in order to facilitate the metastatic process. Activation of the EMT programme suppresses proteins, such as E-Cadherin, involved in cell adhesion whilst upregulating proteins involved in invasion, such as MMPs, and migration, such as vimentin. Indeed, contrary to epithelial cells, which reside in tight colonies with strong cell-cell and cell-substratum adhesion, mesenchymal cells possess several of the key attributes which facilitate tissue invasion and migration, in addition to apoptotic resistance and immunosuppressive functions, which ensure their survival during the invasion-metastasis cascade.

The observation that a high proportion of metastases are differentiated, often reflecting the heterogeneity of the corresponding primary tumour without an apparent mesenchymal phenotype, seems to contradict the notion that secondary tumours arise from disseminated mesenchymal-like cells (Fornoi et al., 2012). However, this apparent paradox has been
explained by the fact that the epigenetic mechanisms which govern EMT, in many cases, are reversible. Hence, tumour cells may undergo transient transitions, initially activating EMT to escape from the primary tumour, invade, disseminate and seed at distant sites where they are then believed to re-differentiate via the reverse transition, MET, in order to colonise and form macrometastases (Brabletz, 2012). However, this process does not account for the existence of undifferentiated metastases. In order to address this, Thomas Brabletz (2012) recently proposed a simplified classification of two principle types of metastasis formation, termed plasticity type I metastasis and genetic type II metastasis. Plasticity type I metastasis corresponds to those with a differentiated phenotype, generated by highly plastic cells capable of activating both EMT and MET as a result of epigenetic modifications triggered by environmental and contextual signals. Conversely, genetic type II metastases present with an undifferentiated phenotype whereby the cells remain in a permanent mesenchymal-like state and only weak differentiation is possible/necessary for the formation of macrometastases. These are believed to arise as a result of an irreversible EMT governed by cell intrinsic properties and permanent genetic alterations. Hence, the key differences between the two proposed metastases are the cause of the EMT, which therefore dictates whether or not it is reversible (Brabletz, 2012). As the events governing plasticity type I metastasis are more common and consequently, better understood, this introduction will mainly focus on the triggers, mechanisms and evidence of the EMT and MET events involved in plasticity type I. For more information on genetic type II metastasis, please refer to the article by Thomas Brabletz (2012).

1.6.3.4 Induction of EMT in Carcinoma Cells

Irrespective of the physiological or pathological outcome, epithelial to mesenchymal transitions are very similar at the cellular level, as they are triggered by like stimuli and are governed by analogous effector molecules, signalling pathways and regulators. The EMT programme involves a complex series of events, requiring significant cross-talk among EMT-inducing signals, several interacting transcription factors and a complex regulatory network consisting of multiple positive and negative feedback loops (Garcia de Herreros and Baulida, 2012; Foroni et al., 2012). Indeed, the transition from an epithelial cell type to a phenotypically distinct, mesenchymal-like cell, capable of synthesising and secreting new growth factors, ECM proteins and MMPs, is not a simple task.
Several stimuli have been shown to induce EMT in cancer cells. These include paracrine growth factor signalling, tumour-stromal cell interactions, hypoxic conditions and even chemotherapy (Cannito et al., 2008; Polyak and Weinberg, 2009; Foroni et al., 2012; Sun et al., 2012). Tumour-associated stroma is believed to contribute significantly to EMT induction. In fact, in some circumstances, tumours are believed to recruit different cell types (fibroblasts, macrophages etc.) into the surrounding stroma which activate the stroma to induce EMT via heterotypical signalling to the cancer cells (Polyak and Weinberg, 2009; Foroni et al., 2012). Several secreted molecules including extracellular matrix components and growth factors such as epidermal growth factors (EGF), hepatocyte growth factors (HGF), fibroblast growth factors (FGF), platelet derived growth factors (PDGF), Insulin-like growth factors 1 and 2 (IGF-1 and -2) and members of the TGF-β family have been shown to induce EMT in different cell types (Hollier et al., 2009; Kalluri and Weinberg, 2009; Foroni et al., 2012). These molecules trigger EMT via the functional activation of a series of EMT-associated transcription factors including Snail1, Snail2 (aka Slug), zinc-finger E-box-binding homobox 1 and 2 (ZEB1 and ZEB2), Twist, Goosecoid and FOXC2 which act pleiotropically to choreograph the EMT programme (Haraguchi, 2009; Das et al., 2009; Yang et al., 2004; Li and Zhou, 2011; Bolos et al., 2002; Casas et al., 2011; Savagner et al., 1997; Kalluri and Weinberg, 2009).

Several signal transduction pathways including Notch, Hedgehog, Integrin and Wnt signalling have been shown to coordinate EMT programmes, some of which are developmental pathways. The involvement of these pathways in EMT provides another link between EMT and stemness (Bailey et al., 2007; Foroni et al., 2012).

Loss of E-Cadherin is considered a hallmark of EMT. Indeed, several of the EMT-inducing transcription factors activate EMT via repression of E-Cadherin either directly, such as Snail1 and Slug, or indirectly, such as Twist. Snail1 is rapidly expressed during the EMT process and has been shown to be directly induced by Notch signalling in response to TGF-β. Furthermore, upregulation of Snail1 was shown to be required for induction of additional E-Cadherin-repressing transcription factors (Matsuno et al., 2012). As inhibition of Snail1 was not sufficient to promote upregulation of E-Cadherin, it has been suggested that while Snail1 initiates the process of CDH1 (the E-Cadherin gene) repression, ZEB1 is required for the full inhibition (Garcia de Herreros and Baulida, 2012). In a similar manner, Twist-induced EMT has been shown to be mediated by Slug (Casas et
Therefore, it is evident that distinct transcription factors are required to interact in order to induce and execute EMT in different contexts.

Recently, micro RNAs have been shown to play a vital role in the maintenance of an epithelial phenotype and reciprocal interactions between EMT-inducing transcription factors and micro RNAs have been shown to be critical in cellular plasticity. For example, a reciprocal feedback loop between the ZEB family of EMT-inducers and the micro RNA 200 family (miR-200) is critical in the induction of EMT and MET. Within the ZEB-miR-200 feedback loop, ZEB inhibits the transcription of miR-200 thereby repressing epithelial differentiation and promoting EMT and a stem-like state, whereas, miR-200 family members inhibit the translation of ZEB, thereby inhibiting the induction of EMT and promoting epithelial differentiation via MET. Therefore, the dynamics of this feedback loop are critical for the invasion-metastasis cascade, inducing EMT for the invasion and dissemination of the cancer cells and inducing MET for metastatic colonisation and macrometastasis formation. The same principle is followed in a second reciprocal feedback loop between miR-34 and Snail1. In this mechanism, Snail1 induces mesenchymal and stem-like characteristics via EMT whereas, miR-34 induces MET and consequently promotes drug sensitivity (Brabletz, 2012). In these examples, both factors control the expression of one another hence, target their own repressors.

Recently, Chang et al., 2011, revealed a link between p53 and the control of EMT when they demonstrated that the transcription of the miR-200 and miR-34 families were induced by p53 (Chang et al., 2011). This finding has enormous implications as it demonstrated that one of the most critical tumour suppressors plays a crucial role in the regulation of phenotypic plasticity (Brabletz, 2012). Furthermore, it linked the most important gene alteration in cancer (mutations in p53) to the emerging fields of cancer stem cells and EMT and their significance for metastasis (Schubert and Brabletz, 2011).
1.6.3.5 Evidence for the Occurrence of EMT in Cancer

There is a wealth of evidence to support the involvement of EMT in cancer progression and metastasis. Several of the biomarkers used to demonstrate EMT have been observed in cancers and have also been shown to correlate with disease progression and a poor prognosis.

In 2004, Yang et al., revealed that the EMT-associated transcription factor Twist, an indirect repressor of E-Cadherin, plays an essential role in tumour metastasis as suppression of Twist in highly metastatic mammary carcinoma cells specifically inhibited metastasis and high levels of Twist expression correlated with human invasive lobular breast carcinoma, a highly infiltrating tumour type associated with loss of E-Cadherin expression (Yang et al., 2004). In addition to this, expression levels of Twist have been shown to positively correlate with Gleason grading and metastasis in human prostate cancer (Kwok et al., 2005). Furthermore, Twist has been shown to inhibit apoptosis in cancer cells and it is thought to play a central role in the resistance to microtubule disrupting agents (Wang et al., 2004). Additional studies have also correlated the presence of critical EMT drivers, such as Snail1 and the ZEB family, with tumour progression, relapse and poor survival (Fuchs et al., 2002; Peinado et al., 2007).

Loss of E-Cadherin is a hallmark of EMT. Several studies have correlated a loss of this molecule with a decrease in patient survival (Hirohashi, 1998). In addition, cell lines with low/no E-Cadherin expression have been shown to demonstrate increased tumourigenicity and metastasis when transferred into immunodeficient mice (Birchmeier and Behrens, 1994). Furthermore, mutations in the E-Cadherin gene have been identified in cancer cells which have been shown to be more susceptible to EMT and metastasis (Kalluri and Weinberg, 2009).

Evidence of EMT can be observed in cancer tissues based on the expression levels of different Cadherins. The so-called “Cadherin switch” from E-Cadherin to N-Cadherin, which is expressed by mesenchymal cells, neural tissue, fibroblasts and cancer cells, has often been used to monitor the progression of EMT during embryonic development and cancer progression (Zeisberg and Neilson, 2009). Gravdal et al., (2007) revealed that Cadherin switching (from E-Cadherin to N-Cadherin) observed in prostate cancer tissue microarrays showed strong and significant associations with multiple end points of progression and cancer-specific death (Gravdal et al., 2007). Hence, provided evidence for
the contributory role of EMT in cancer progression and its association with a poor clinical outcome. In support of these findings, a recent study, conducted by Tanaka and colleagues, demonstrated a clear link between the expression of N-Cadherin and invasive, metastatic, castration resistant prostate cancer (CRPC). In this study, N-Cadherin-specific antibodies were shown to delay the progression to castration resistance, inhibit invasion of surrounding tissues, suppress tumour growth and reduce metastasis in castrated mice. Hence, this work provided further support for the critical role of EMT in prostate cancer progression and the potential of immunotherapy as a strategy to combat this disease (Tanaka \textit{et al.}, 2010). However, it is important to note that the Type 3 EMTs, which occur in the context of cancer, may not represent a complete transition from an epithelial to a mesenchymal phenotype, therefore, dual expression of epithelial and mesenchymal Cadherins has also been reported (Lou \textit{et al.}, 1999).

In the context of breast cancer, basal-like tumours, which show high expression of the mesenchymal markers N-Cadherin and vimentin and low expression of E-Cadherin, demonstrate a poor prognosis with a higher risk of metastasis, compared to other invasive breast carcinomas (Foroni \textit{et al.}, 2012). Furthermore, several cell line studies have revealed that the more invasive and metastatic breast cancer cell lines display hallmarks of EMT-derived cells (Hollier \textit{et al.}, 2009).

Circulating tumour cells (CTCs) of primary breast cancer patients have been found to express EMT-associated markers such as vimentin (Kasimir-Bauer \textit{et al.}, 2012). However, CTCs with an EMT signature are likely to have been overlooked in the majority of studies which exploit epithelial markers to purify CTCs from patient blood (Nieto and Cano, 2012).

Several \textit{in vitro} and \textit{in vivo} studies have revealed a correlation between EMT and an invasive and metastatic phenotype. Moreover, evidence of EMT in patient material has been provided based on the expression of EMT-associated transcription factors, mesenchymal-associated markers expressed by CTCs and evidence of Cadherin switching, which correlated with disease progression, metastasis and a poor prognosis. However, despite the wealth of data implicating EMT in cancer progression, there is no direct evidence of this phenomenon occurring in human tumours.
1.6.3.6 Doubts Surrounding the Role of EMT in Cancer Progression

Although several studies have provided evidence to support the role of EMT in cancer progression, a lack of direct evidence in clinical specimens has rendered it an incredibly controversial subject, which, consequently is not accepted by all (Garber, 2008). In his contentious article, Tarin (2005) argued that there was insufficient evidence to support a role of EMT in neoplasia as “In >40 years of experience of reporting the pathology of many thousands of tumor samples, the author has not seen convincing evidence of EMT...” In addition, Tarin argued that EMT cannot be a significant event in carcinogenesis and tumour progression due to a lack of extensive documentation by other surgical pathologists and because it (EMT) isn’t used to score cancers (Tarin, 2005). The occurrence of epithelial to mesenchymal transition in cancer can account for several of the previously unexplained phenomena associated with disease progression. Therefore, to disregard the possibility that a known biological process (EMT) may be reactivated in cancer cells, a concept supported by more than 15 years of research and a large body of in vitro and in vivo data, requires the proposal of an alternative hypothesis which may better address the phenomena observed and account for the successful completion of the invasion-metastasis cascade. However, to date, a more convincing hypothesis, suggesting the mechanisms employed for the successful completion of the invasion-metastasis cascade, has yet to be presented. Furthermore, the inability to observe EMT in clinical specimens can be explained by the fact that 1) EMT is a transient process which may be overlooked 2) there are currently no markers which distinguish cancer cells which have transitioned through EMT from the mesenchymal cells of the tumour stroma 3) EMT may be an extremely rare event, supported by the inefficiency of metastasis and 4) metastasising EMT cells are believed to revert to an epithelial form via mesenchymal to epithelial transition (MET) in order to colonise at secondary sites thereby eliminating any evidence of a previous passage through EMT (Foroni et al., 2012).

Nonetheless, despite controversy it is widely accepted that epithelial to mesenchymal transition, and the stem-like properties which accompany this change in phenotype, of differentiated cancer cells can strongly enhance tumour cell dissemination (Brabletz, 2012).
1.6.3.7 Cancer Stem Cells and EMT

In 2008, Mani et al., combined two of the emerging fields in cancer biology by revealing a link between EMT and cancer stem cells when they demonstrated the acquisition of stem-like properties (enhanced sphere formation, self-renewal and a change in antigenic profile) by transformed human mammary epithelia artificially induced to undergo EMT. Furthermore, this group demonstrated that both normal and putative breast cancer stem cells, with the antigenic profile CD44$^{hi}$CD24$^{lo}$, expressed several of the EMT-associated markers at the mRNA level (Mani et al., 2008).

Since the release of this paper, several groups have implicated EMT in the generation of cancer stem-like cells (Fazul et al., 2009; Santisteban et al., 2009; Kong et al., 2010). It is however, important to note that these studies demonstrated the generation of stem-like cells via EMT of differentiated cancer cells. Therefore, the cells generated via EMT must be distinct from the original tumour initiating cell. This notion is supported by the fact that the EMT marker profile sometimes contradicts the profile of the CSCs from the same tissue. For example, prostate cancer stem cells are postulated to express high levels of the transmembrane receptor integrin $\alpha_2\beta_1$, (Collins et al., 2005) however, EMT-derived cells from the prostate cancer cell line OPCT-1 did not demonstrate expression of this protein (Unpublished data – See Chapter 5).

The cancer stem cell hypothesis states that the cancer stem cell is solely responsible for tumour initiation, tumour differentiation, tumour maintenance, tumour spread (metastasis) and tumour relapse following therapy. As is evident, EMT-derived cells cannot fulfil the cancer stem cell criteria as they are not implicated in initiation of the primary tumour. However, there is much evidence to support the role of EMT-derived cells in metastasis and chemotherapeutic resistance (Zhau et al., 2008; Kyprianou et al., 2010; Tanaka et al., 2010). Thus, it is plausible that, contrary to the CSC hypothesis, the progeny of transformed stem cells are also tumourigenic and that such progeny may undergo EMT which then confers stem-like properties and the ability to invade and metastasise. Therefore, whereas the adult tissue stem cell may be the initial site of malignant transformation, their transitioning progeny (differentiated cancer cells undergoing EMT), rather than the cancer(ous) stem cells themselves may be accountable for invasion and metastasis. Therefore, the term “cancer stem cell” may be considered to refer to a number of different cell populations including transformed stem/progenitor cells and EMTs, rather
than one “super-malignant” cell population, all of which have a role to play in tumourigenesis.

The acquisition of stem-like properties by metastatic cancer cells which have activated the EMT programme addresses several of the conceptual dilemmas in cancer biology. Firstly, both cancer stem-like cells and EMTs are known to resist apoptosis and evade immune destruction (See Section 1.6.3.8), properties which may account for the survival of metastatic cells during the invasion-metastasis cascade. Secondly, the stem-like characteristics of enhanced proliferative potential, differentiation and self-renewal ability may explain the formation of differentiated macroscopic metastases in the case of plasticity type I metastasis.

Figure 1.14 illustrates the possible events involved in the invasion-metastasis cascade in plasticity type I metastasis taking cancer stem cells, EMT-derived cancer stem-like cells and circulating tumour cells (also EMT derived) into consideration.

In 2010, Battula et al., revealed that cells which had transitioned via EMT exhibited characteristics of mesenchymal stem cells (MSCs) as the EMT-derived cells demonstrated a MSC gene signature, the ability to migrate towards cells in vitro and wounds in vivo and multilineage differentiation potential (Battula et al., 2010). Generation of MSCs via EMT would account for the existence of more than one “cancer stem cell” population in tumours and may also explain several of the phenomena observed in cancer. For example, MSCs are known to possess multiple immunosuppressive functions, chemotherapeutic resistance mechanisms and resistance to apoptosis which would certainly assist with the completion of the invasion-metastasis cascade (Selmani et al., 2008; Szegezdi et al., 209; Roodhart et al., 2011; Ertas et al., 2012; Houthuijzen et al., 2012).

While the original cancer stem cell hypothesis may address tumour initiation and other aspects of tumourigenesis, it is evident that it should be revised, taking EMT into consideration for the generation of a distinct population of cancer stem-like cells which contribute significantly to tumour progression. It is evident that both the tumour-initiating cancer stem cells and those generated via EMT have serious therapeutic implications and must be targeted in order to prevent tumour progression and reoccurrence.
**Figure 1.14:** Illustrating the steps of the invasion-metastasis cascade for plasticity type I metastasis. The process begins when the tumour associated stroma or tumour cells release signals, activating the generation of invasive and metastatic stem-like cells via EMT which escape from the primary tumour, invade the surrounding stroma, penetrate local vessels (intravasation) and disseminate as circulating tumour cells (CTCs) prior to penetrating the vessel wall at distant sites (extravasation), reverting to an epithelial form via MET and colonising to form macrometastases. (EMT – epithelial to mesenchymal transition, MET – mesenchymal to epithelial transition, CSC – cancer stem cell, CTC – circulating tumour cell). (Adapted from Foroni et al., 2012).
1.6.3.8 Therapeutic Implications of EMT and CSCs.

The tumour-initiating cancer stem cells and the cancer stem-like cells generated through EMT represent an important clinical challenge. Through a variety of inherent and acquired traits, both cell types have been shown to resist chemotherapy, radiotherapy and immunotherapy. In fact, by targeting only the bulk of the tumour, conventional therapies often enrich for the cancer stem/stem-like cells, as shown in clinical studies of breast cancer, where the proportions of CD44+/CD24-low CSC/EMT-derived cells increased significantly after chemotherapy (Li et al., 2008).

A number of traits account for the increased resistance of cancer stem/stem-like cells to therapy, the majority of which are also common to their normal stem cell counterparts. Conventional chemo/radiotherapies mainly target rapidly dividing cells, as such, the inherent quiescent and slow cycling nature of CSCs renders them resistant to radiotherapy and several of the first-line chemotherapeutic agents. In addition, increased expression of multi-drug resistant proteins and related members of the ATP-binding cassette (ABC) transporter family, provide further protection by facilitating the efflux of drugs (Alison et al., 2012). Furthermore, high ALDH activity, observed in CSCs, has been shown to assist with drug metabolism, thereby providing cancer stem/stem-like cells with an additional defensive mechanism (Hollier et al., 2009). Failing these defences, cancer stem/stem-like cells also demonstrate efficient DNA repair mechanisms, increased expression of anti-apoptotic proteins and downregulation of pro-apoptotic genes hence, are highly resistant to apoptosis (Qi et al., 2012; Liu et al., 2006; Conticello et al., 2004) (See Section 1.5.5.5.5).

The cancer stem-like cells generated via EMT have been shown share several properties with mesenchymal stem cells (Battula et al., 2010). As such, it is possible that these cells may provide further therapeutic resistance via secretion of antiapoptotic factors, secretion of fatty acids which protect against chemotherapy, and resistance to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor mediated apoptosis (Szegezdi et al., 2009; Roodhart et al., 2011; Houthuijzen et al., 2012)

As previously discussed, both cancer stem cells and EMT-derived stem-like cells possess a variety of mechanisms which enable immune-avoidance. Such mechanisms include downregulation of MHC class I molecules, expression and secretion of HLA-G and secretion of immunosuppressive cytokines (Brown et al., 2012; Morandi et al., 2012; Qi et al., 2012). (See section 1.5.5.5.4).
These populations present a serious dilemma to cancer biologists and oncologists as conventional therapies are thought to only target the bulk of the tumour, leaving the resistant CSCs and EMT-derived cancer stem-like cells behind to repopulate the tumour, hence the disease will inevitably progress. To achieve full and permanent eradication of tumours, it is necessary to further our understanding of the resistant populations in order to design CSC/EMT-specific therapies which should be used in combination with therapies targeting the rapidly-dividing bulk of the tumour. Indeed, the development of cancer stem cell-specific therapies is currently underway (Foroni et al., 2012; OncoMed Pharmaceuticals, 2012). Furthermore, treatments targeting the molecular activators of EMT are hoped to prevent metastasis and reduce the generation of resistant, stem-like cells (Hollier et al., 2009). In addition, therapies targeting the epigenetic changes governing EMT aim to revert EMT-derived cells to a more differentiated, more sensitive epithelial form (Brabletz, 2012). Together, these therapies hope to overcome the challenges faced by therapeutic resistance and offer a permanent cure.

1.7 Rationale and Outline of the Study

Cancer stem cells (CSCs) and epithelial to mesenchymal transition (EMT) have recently emerged as important concepts in cancer biology. Once considered separate phenomena, these two areas were recently combined when Mani et al., (2008) demonstrated that EMT generates cells with the properties of stem cells. However, as these areas of research remain in their infancy, the current literature presents several conflicting hypotheses. Furthermore, many of the studies investigating the relationship between EMT and CSCs have used breast cancer as a model hence, further studies investigating this relationship are required in the prostate cancer field.

In 2005, Collins and colleagues isolated putative prostate cancer stem cells by exploiting the markers they had previously identified for normal prostate stem cells. The present study initially aimed to follow the method established by Collins et al., (2005), in an attempt to isolate and characterise putative prostate cancer stem cells from primary cultures and prostate cancer cell lines, with a view to investigating their immunological status and determining whether or not these cells could provide viable targets for immunotherapy. However, using the methods established by Collins et al., (2005), this study failed to identify and isolate putative prostate cancer stem cells. Consequently, the focus of the
study shifted to investigating the properties of epithelial to mesenchymal transition and exploring a possible link between EMT and the generation of CSCs in human prostate cancer.
Chapter 2:

Materials and Methods

2.1 Laboratory Consumables and Equipment

2.1.1 Reagents

All reagents were stored according to the manufacturer’s instructions and used within the expiry date.

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Pen/strep antibiotic solution
Non-Essential Amino Acids (NEAA)
Sodium Pyruvate
Stem Cell Factor (SCF)
Testosterone

**Other Cell Culture Materials**

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**Chemical Reagents**

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<tr>
<td>[3H]-Thymidine</td>
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<tr>
<td>Acrylamide</td>
<td>Geneflow</td>
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<tr>
<td>Ammonium Persulphate (APS)</td>
<td>Geneflow</td>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Chloroform</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Copper phthalocyanine-3,4’,4”,4”-tetrasulfonic acid tetrasodium salt</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Crystal violet</td>
<td>Fisher Scientific</td>
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<tr>
<td>DAPI VECTASHIELD® Mounting media</td>
<td>Vector Labs</td>
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<tr>
<td>Deoxyribonucleotide Triphosphahte (dNTP)</td>
<td>Bioline</td>
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<tr>
<td>Dithiothreitol (DTT)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Docetaxel</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Ethanol</td>
<td>BDH</td>
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<tr>
<td>Ethyldiamine Tetraacetic Acid (EDTA)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Glycerol</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Hydrochloric acid (HCl)</td>
<td>Fisher Scientific</td>
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<tr>
<td>Isopropanol</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Isoton</td>
<td>Beckman Coulter</td>
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<tr>
<td>Liquid nitrogen</td>
<td>BOC</td>
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</table>
Marvel milk powder  
Methanol  
Microscint-O scintillation fluid  
MTT  
NANOpure Diamond distilled water  
Paraformaldehyde 96%  
PBS tablets  
Phenol/Chloroform/Isomyl alcohol  
Phosphate Buffered Saline (PBS) tablets  
Presept  
Propidium Iodide (PI)  
RapidStep ECL Reagent  
Ribonuclease (RNase)  
RNA-STAT-60  
Sodium chloride (NaCl)  
Sodium dodecyl sulphate (SDS)  
Stacking gel buffer for SDS gels  
SYBR® Green Supermix  
Teepol  
TEMED  
Tris-base  
Tween-20  
Ultrapure 10X Tris/Glycine/SDS  
Ultrapure 10X Tris/Glycine  
Western-C Precision Plus molecular weight marker  

**Immunochemical Reagents**

Primary antibodies

Mouse anti-human HLA-G (5A6G7)  
Mouse anti-human P63  
Mouse anti-human Fibronectin (IST-9)  
Mouse anti-human AR  
Rabbit anti-human S100A4 (FSP1)  

Supplier

Premier Brands  
Fisher Scientific  
Perkin-Elmer  
Sigma Aldrich  
Barnstead  
Sigma Aldrich  
Sigma Aldrich  
Oxoid  
Johnson and Johnson  
Sigma Aldrich  
Calbiochem  
Quiagen  
AMS Biotechnology  
Fisher Scientific/ Sigma  
Sigma Aldrich  
Geneflow  
BioRad  
Geneflow  
Melford  
Sigma Aldrich  
Geneflow  
Geneflow  
BioRad

Abcam  
Abcam  
Abcam  
Abcam  
Abcam
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<tr>
<th>Antibody Description</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Rabbit anti-human vimentin (RV202)</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rabbit anti-human CD133</td>
<td>Abcam</td>
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<tr>
<td>Mouse IgG isotype negative control antibody</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>Mouse anti-human β-Catenin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse anti-human E-Cadherin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse anti-human N-Cadherin</td>
<td>BD Biosciences</td>
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<tr>
<td>Mouse anti-human CD44 (156-3C11)</td>
<td>Cell Signalling Technology</td>
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<tr>
<td>Rabbit anti-human Sox2 (D6D9) XP(R)</td>
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<tr>
<td>Rabbit anti-human Oct4</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Rabbit anti-human β-actin antibody</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Rabbit anti-human Nanog (D73G4) XP(R)</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Rabbit anti-human Survivin (71G4B7)</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Rabbit anti-human HER1</td>
<td>Epitomics</td>
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<tr>
<td>Mouse anti-human E-Cadherin</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Mouse anti-human VLA-2 (α2β1 integrin)</td>
<td>Millipore</td>
</tr>
<tr>
<td>Rabbit IgG isotype negative control antibody</td>
<td>Pierce Biotechnology</td>
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<tr>
<td>Goat anti-human SNAI1 (E-18)</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Goat anti-human SLUG (D-19)</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Goat anti human CD24</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Goat anti human PSCA</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Rabbit anti-human twist (H-81)</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Rabbit anti-human CD24 (FL-80)</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Mouse anti-human cytokeratin pan</td>
<td>Sigma Aldrich</td>
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</table>

**Secondary antibodies**

<table>
<thead>
<tr>
<th>Antibody Description</th>
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</thead>
<tbody>
<tr>
<td>Steptavidin-HRP secondary antibody</td>
<td>Biorad</td>
</tr>
<tr>
<td>Goat anti-mouse-HRP secondary antibody</td>
<td>Dako</td>
</tr>
<tr>
<td>Rabbit anti-goat-HRP secondary antibody</td>
<td>Dako</td>
</tr>
<tr>
<td>Swine anti-rabbit-HRP secondary antibody</td>
<td>Dako</td>
</tr>
<tr>
<td>Chicken anti-rabbit Alexafluor®488</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Donkey anti-goat Alexafluor®488</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Donkey anti-goat Alexafluor®568</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Goat anti-rabbit Alexafluor®488</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Goat anti-mouse Alexafluor®488</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
Goat anti-rabbit Alexafluor®568 Invitrogen
Goat anti-mouse Alexafluor®568 Invitrogen

Conjugated antibodies
Mouse anti-human CD44-FITC AbD Serotec
Mouse IgG-FITC isotype negative control antibody AbD Serotec
Mouse anti-human CD157-FITC AbD Serotec
Mouse anti-human Vimentin (clone RV202)-PE BD Bioscience
Mouse IgG1k-PE isotype control BD Bioscience
Mouse anti-human CD45-APC Alexafluor®750 Beckaman Coulter
Mouse anti-human CD105-PE (SN6) eBioscience
Mouse anti-human CD90 (Thy-1)-APC (SE10) eBioscience
Mouse anti-human CD73-EFluor®450 (AD2) eBioscience
Mouse anti-human CD133/1-APC MACS
Mouse anti-human CD133/2-PE MACS
Iso mouse IgG1a-APC MACS
Iso mouse IgG2b-PE MACS

Kits Supplier

Stem Cell Assay:
ALDEFLUOR™ Stem Cell Technologies

Protein Assay:
Bicinchoninic Acid Kit Sigma Aldrich

Cell Invasion:
Biocoat Matrigel™ Invasion Chamber (8μm pore) BD

Proliferation Assay:
ViaLight® Plus proliferation assay kit Lonza
### 2.1.2 Equipment

All glass items were washed in dilute presept/teepol and rinsed twice in distilled water prior to use. Please refer to Appendix II for the complete list of equipment used.

**Laboratory plastics, glassware and sharps**

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>0.1 µm pore size filters</td>
<td>Starstedt</td>
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<tr>
<td>0.2 µm pore size filters</td>
<td>Sartorius</td>
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<tr>
<td>3 ml syringes</td>
<td>Stem Cell Technologies</td>
</tr>
<tr>
<td>6, 24, 96-well tissue culture plates</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>96 well black/clear, tissue culture treated imaging plate</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>96 well microtiter plate</td>
<td>Nunc, Denmark</td>
</tr>
<tr>
<td>96-well microtiter plate</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>96-well round-bottom culture plates</td>
<td>Porvair advanced mats</td>
</tr>
<tr>
<td>96-well white microplate</td>
<td>Sterlin</td>
</tr>
<tr>
<td>Bijou Tubes (7 ml)</td>
<td>Stem Cell Technologies</td>
</tr>
<tr>
<td>Blunt end needles</td>
<td>Sarstedt UK</td>
</tr>
<tr>
<td>Cell culture flasks (T25, T75, T175 cm³)</td>
<td>BD Falcon</td>
</tr>
<tr>
<td>Cell strainers</td>
<td>BD Biocoat™</td>
</tr>
<tr>
<td>Collagen dishes (35 mm, 10 mm)</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Collagen cell culture flasks (T25, T75 cm³)</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Companion plates</td>
<td>SLS</td>
</tr>
<tr>
<td>Coverslips</td>
<td>Pyrex</td>
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<tr>
<td>Conical flasks (50 ml, 100 ml)</td>
<td>TPP</td>
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<tr>
<td>Cryovials (1.5 ml)</td>
<td>Zeiss, Germany</td>
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<tr>
<td>Duplex dish</td>
<td>Sarstedt</td>
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<tr>
<td>Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml)</td>
<td>Sarstedt</td>
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<tr>
<td>Flow cytometry tubes</td>
<td>Tyco healthcare group</td>
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<tr>
<td>Falcon tubes (50 ml, 15 ml)</td>
<td>Sarstedt</td>
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<td>Filter tips (0.5-10 µl, 2-20 µl, 20-200 µl, 200-1000 µl)</td>
<td>Greiner bio-one/Sarstedt</td>
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<tr>
<td>Flat-bottom culture dishes (6 well/ 24 well)</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Glass coverslips</td>
<td>SLS</td>
</tr>
<tr>
<td>Nitrocellulose Western blot membrane</td>
<td>GE Water and Process Technologies</td>
</tr>
</tbody>
</table>
LS columns | Miltenyi  
---|---  
Micro tips (0.5-10 µl, 20-200 µl, 200-1000 µl) | Sarstedt  
MS Columns | Miltenyi  
Measuring cylinders (0-10 ml, 0-100 ml, 0-500 ml, 0-1000 ml, 0-2000 ml) | Pyrex  
Pasteur pipettes | Sarstedt  
Petri dishes | Sterilllin  
Pipettes (5 ml, 10 ml, 25 ml) | Sarstedt  
Plastic Pasteur pipettes | Sarstedt  
Pre separation filters | Miltenyi  
Real-time qPCR tubes | Qiagen/ Axygen Scientific  
Scalpels | Swann Morton/TPP  
Screw-top tubes (15 ml) | Sarstedt  
Sealing tape (Nescofilm) | Bando Chemicals  
Serological pipettes | Sarstedt  
Syringes (10 ml, 20 ml) | Becton Dickenson  
T25/T75/T175cm³ culture flasks | Sarstedt  
Tissue culture dishes (35 mm) | Stem Cell Technologies  
Ultra low adherent 24 well/ 96 well plates | Corning  
Universal tubes (20 ml) | Sterilllin  
Unifilter 96-well filter plates | Perkin-Elmer  
Western blot filter paper | Schleicher-Schwell  
Amersham X-ray film | G.E. Healthcare  
0.2 µm filters | Sartorius

**Equipment**

**Item**

2100 Bioanalyzer | Agilent Technologies  
CCD Camera | Fuji Systems  
Coulter Counter | Beckman Coulter  
Cryostat | Leica  
Electrophoresis Gel Tanks | Geneflow  
Filtermate Harvester | Perkin Elmer
<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
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<tr>
<td>Gallios Flow Cytometer</td>
<td>Beckman Coulter</td>
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<tr>
<td>iCycler I Q Multicolour RT PCR Machine</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Leica TCS SP5 Confocal Microscope</td>
<td>Leica</td>
</tr>
<tr>
<td>PALM Laser-capture microdissector (LCM)</td>
<td>Carl Zeiss</td>
</tr>
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<td>Micropipettes (2 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)</td>
<td>Genex/ Beta/ Gilson/ Sealpette</td>
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<td>Microplate Luminometer</td>
<td>Berthold Detection Systems</td>
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<td>Midi MACS magnet</td>
<td>Miltenyi</td>
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<tr>
<td>Mini MACS magnet</td>
<td>Miltenyi</td>
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<td>MoFlo™ XDP High-Speed Cell Sorter</td>
<td>Beckman Coulter</td>
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<tr>
<td>Nanodrop ND-800 spectrophotometer</td>
<td>Thermo Scientific</td>
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<td>Nanopure Diamond water reservoir</td>
<td>Barnstead</td>
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<tr>
<td>Nikon Eclipse Ts100 Light Microscope</td>
<td>Olympus</td>
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<tr>
<td>NXT Top-Count Microplate Scintillation Counter</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>Octo MACS magnet</td>
<td>Miltenyi</td>
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<tr>
<td>Olympus BX51 Fluorescence Microscope</td>
<td>Olympus</td>
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<td>Orbital Incubator</td>
<td>Stuart</td>
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<tr>
<td>Plate reader</td>
<td>Biorad and Tecan</td>
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<td>96-well plate harvester</td>
<td>Packard</td>
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<td>Real-time qPCR thermal cycler</td>
<td>Qiagen</td>
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<tr>
<td>Sonicator</td>
<td>VWR</td>
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<tr>
<td>Tecan Ultra Microtiter Plate Reader</td>
<td>Tecan</td>
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<tr>
<td>Transfer apparatus</td>
<td>Geneflow</td>
</tr>
<tr>
<td>Top count scintillation counter</td>
<td>Packard</td>
</tr>
<tr>
<td>UNO-thermoblock</td>
<td>Biometra</td>
</tr>
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</table>
2.1.3 Buffers and Stains

Buffers/Stains used for cell culture

*Trypan Blue for enumeration of cells:*
0.1% (v/v) solution Trypan Blue in DPBS.

*MACS Buffer:*
DPBS
2 mM EDTA
0.5% (w/v) BSA
pH adjusted to 7.2
Sterile-filtered and de-gassed by sonication for 15 minutes prior to use.

*Crystal Violet Stain:*
0.5% (w/v) Crystal Violet
70% (v/v) Ethanol

Buffers/Stains used for western blot analysis

*Total Cell Lysis Buffer:*
50 mM Tris
5 mM EDTA
1% (w/v) SDS

*1x 8% Resolving SDS gel:*
2300 μl ddH₂O
1300 μl Acrylamide
1300 μl 1.5M Tris-HCl (pH 8.8)
50 μl 10% (w/v) SDS
50 μl 10% (w/v) APS
3 μl TEMED

*1x10% Resolving SDS gel:*
1900 μl ddH₂O
1700 μl Acrylamide
1300 μl 1.5M Tris-HCl (pH 8.8)
50 μl 10% (w/v) SDS
50 μl 10% (w/v) APS
3 μl TEMED
**1x 5% Stacking SDS gel:**

- 1400 µl ddH₂O
- 330 µl Acrylamide
- 250 µl 1M Tris-HCL (pH 6.8)
- 20 µl 10% (w/v) SDS
- 20 µl 10% (w/v) APS
- 2 µl TEMED

**Reducing sample buffer:**

- 0.5 M Tris-HCl (pH 6.8)
- 2% (w/v) SDS
- 10% (v/v) Glycerol
- 1% DTT

ddH₂O to final volume

**Deionised phosphate buffered saline+ TWEEN-20 (DPBS-0.1% (v/v) TWEEN):**

- 1x DPBS
- 0.05% TWEEN-20

**DPBS-0.1% (v/v) TWEEN+ Marvel blocking buffer:**

- 3% (w/v) Marvel Milk powder
- 100 ml DPBS-0.1% (v/v) TWEEN

**Tris/Glycine/SDS Gel Running Buffer:**

- 10x Tris/Glycine/SDS (0.25 M Tris, 1.92 M Glycine, 1% (w/v) SDS)

Made to 1x with ddH₂O

**Tris/Glycine Gel Transfer Buffer:**

- 100 ml 10x Tris/Glycine
- 200 ml Methanol
- 700 ml ddH₂O
Buffers used for flow cytometry analysis

*Fixation solution:*
1% (w/v) paraformaldehyde in DPBS

*Permeabilisation solvent:*
100% Methanol

*Flow cytometry buffer:*
0.5% (w/v) BSA
1x DPBS

Buffers for Immunofluorescence

*Fixation solution*
4% (w/v) paraformaldehyde
1x DPBS

*Deionised phosphate buffered saline + TWEEN-20 (DPBS-0.1% (v/v) TWEEN):*
As previously

*Blocking solution*
1x DPBS-0.1% (v/v) TWEEN
10% (w/v) BSA

2.1.4 Primary Cell Culture Media/Solutions/Feeder Cells

Transport Media for Transport of Primary Tissue
RPMI1640
5% (v/v) FCS

Prepared, aliquotted and stored at the hospital prior to surgery. If the tissue was to be retained overnight, 10,000 IU of the protease inhibitor aprotinin was added per 500 ml media.
Primary Epithelial Growth (PEG) Media for Culture of Prostate Epithelia

500 ml Keratinocyte serum-free media (KSFM)
2.5 μg epidermal growth factor human recombinant protein (EGF)
25 mg bovine pituitary extract (BPE)
1 μg leukaemia inhibitory factor (LIF)
1 μg stem cell factor (SCF)
50 μg cholera toxin (CT)
0.5μg granulocyte-macrophage colony stimulating factor GM-CSF
5 ml L-glutamine
5 ml pen/strep antibiotic solution
The supplements were added to 5 ml KSFM which was sterile-filtered prior to combining with the remaining 495 ml of KSFM. The media was refrigerated at 4°C between use.

RPMI 1640 plus 10% (v/v) FCS (R10)
500 ml of RPMI1640
50 ml FCS
5 ml L-glutamine
5 ml pen/strep antibiotic solution
The media was refrigerated at 4°C between use.

0.3% (w/v) BSA in 1x DPBS
Prepared in advance in 50 ml aliquots, heat inactivated for 1 hour at 56°C and sterile filtered prior to use. In between use, it was refrigerated at 4°C.

Collagenase solution
Freshly prepared in primary epithelial growth (PEG) medium and R10 (1:1) to a final concentration of 200 IU/ml and sterile-filtered prior to use.

Mouse fibroblast feeder cells (STOs)
Cultured in T175 tissue culture flasks in DMEM plus 10% (v/v) FCS until 70-80% confluent. Then harvested, re-suspended in 35 ml media and irradiated for 10 minutes using a 60 Gy gamma source. These were refrigerated and could be used up to 5 days following irradiation.
2.1.5  Cell Lines and Growth Conditions

All cells were cultured in incubators supplying a 37°C, 5% (v/v) CO₂ atmosphere

*Table 2.1: List of tumour cell lines used in this study, describing their characteristics, source and culture media.*

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Source</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>Androgen independent metastatic prostate cancer</td>
<td>American Type Culture Collection</td>
<td>HAM’S F12 + 10% (v/v) FCS, 2 mM L-Glutamine + 1% (v/v) NEAA</td>
</tr>
<tr>
<td>DU145</td>
<td>Androgen independent metastatic prostate cancer</td>
<td>American Type Culture Collection</td>
<td>DMEM + 10% (v/v) FCS, 2 mM L-Glutamine, 1% (v/v) NEAA + 1% (v/v) Sodium Pyruvate</td>
</tr>
<tr>
<td>P4E6</td>
<td>Androgen independent primary prostate cell line</td>
<td>Professor Norman Maitland (University of York, England)</td>
<td>KSFM + 2% (v/v) FCS + 2 mM L-Glutamine</td>
</tr>
<tr>
<td>OPCT-1</td>
<td>Androgen independent primary prostate cancer cell line</td>
<td>ONYVAX</td>
<td>KSFM + 2% (v/v) FCS + 2 mM L-Glutamine</td>
</tr>
<tr>
<td>OPCT-2</td>
<td>Androgen independent primary prostate cancer cell line</td>
<td>ONYVAX</td>
<td>KSFM + 2% (v/v) FCS + 2 mM L-Glutamine</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>Human Breast adenocarcinoma</td>
<td>American Type Culture Collection</td>
<td>McCoy’s 5A + 10% (v/v) FCS</td>
</tr>
<tr>
<td>NTERA2/D1</td>
<td>Human pluripotent testicular embryonal carcinoma</td>
<td>Dr Neil Cross (Sheffield Hallam University)</td>
<td>DMEM + 10% (v/v) FCS</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>Murine embryonic fibroblasts</td>
<td>American Type Culture Collection</td>
<td>DMEM + 10% (v/v) FCS</td>
</tr>
<tr>
<td>STO</td>
<td>Murine embryonic fibroblasts</td>
<td>American Type Culture Collection</td>
<td>DMEM + 10% (v/v) FCS</td>
</tr>
</tbody>
</table>
2.1.6 Additional Cells/ Tissue/ Mice

*Cord Blood*

Ethically approved, fresh or frozen cord blood was kindly donated from the Anthony Nolan Cell Therapy Centre and was processed immediately at the Anthony Nolan Cell Therapy Centre with the help of the staff. Therefore, no media was used to maintain the cells. Isolated cord blood stem cells were also provided by the Anthony Nolan Cell Therapy Centre in frozen aliquots. These were processed without media.

*Fresh prostate tissue*

Ethically approved, fresh prostate tissues were collected from Nottingham City Hospital following radical prostatectomies and trans-urethral resectioning of the prostate (TURP) procedures and were processed immediately on return to the John van Geest Cancer Research Centre. Isolated cell populations were maintained in media as described in Section 2.1.4.

*Athymic Mice*

Thirty male athymic nude mice, six weeks of age, were kindly provided by Harlan Laboratories, UK. These were maintained in accordance with UK Home Office regulations and allowed to acclimatise for two weeks in the animal facility prior to conducting *in vivo* studies. Mice were kept in IVC cages supplied with laminar air flow and autoclaved food and water taken *ad libitum.*
2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Routine Cell Culture and Cell Counting

The cell lines used for the duration of this project are listed with the required culture conditions in Table 2.1. Cryovials of approximately 1x10^6 cells were stored long-term in liquid nitrogen and initiating a cell line required removal, thawing and washing of cells by centrifugation at 400 g for 3 minutes in 10 ml of appropriate media. The wash step was required for the removal of excess DMSO, an agent used to protect the cells by preventing ice crystal formation during the freezing process. Following centrifugation, cell pellets were re-suspended in 6 ml of the appropriate media, transferred into a T25 culture flask and incubated at 37°C in air atmosphere with 5% (v/v) CO₂. Once cells reached 70-80% confluency the media was poured off, the cells were washed twice with DPBS and they were then harvested from the flask by incubation at 37°C with 1 ml 0.05% trypsin mixed with 0.02% versene (T&V). Following trypsinisation, 10 ml of media was added to the cells in the flask and the cells were transferred into a 20 ml universal tube which was then centrifuged at 400 g for 3 minutes. The pellet was then re-suspended in 10 ml of appropriate media and the cells were split between the original T25 and a new T75 flask in a process known as “passaging”. Each passage was recorded and the use of cell lines in assays with as low a passage as possible was desirable.

Cell counting was performed by re-suspending a harvested cell pellet in 10 ml of appropriate media (or less volume if smaller number of cells was expected) and performing a 1:10 dilution with trypan blue. A haemocytometer was used to enumerate the cells and dead cells, which stained blue, were identified and excluded from the cell count.

2.2.1.2 Fresh Prostate Tissue Processing

2.2.1.2.1 Separation of stroma from epithelia and selection of integrin α2β1-high cells

Using a “Nottingham needle”, 6 cores of prostate tissue were taken at different points from a surgically removed prostate. Three cores were snap-frozen in optimum cutting temperature compound (OCT) and the remaining 3 cores were placed separately in 15 ml of transport media and were stored at 4°C prior to collection. Alternatively, pieces of
tissue from trans-urethral resectioning of the prostate (TURP) procedures were either snap frozen in OCT, preserved in formalin, or placed in transport media for processing.

Providing the surgery was completed and the cores were extracted prior to 3pm, the tissue was collected the same day and was transported on dry ice to our facilities. The cores were then stored at 4°C awaiting processing until 3pm. At 3pm sterile DPBS, Petri dishes, tweezers and scalpels were placed in a laminar flow hood. Each core was processed separately; hence this procedure was executed in triplicate. Sterile tweezers were used to remove the cores from the transport media and place them in sterile Petri dishes where they were washed with 7 ml sterile DPBS and then cut into many small pieces using a scalpel. Subsequently 7.5 ml of freshly prepared collagenase solution was pipetted onto the chopped tissue and the tissue was pipetted up and down before being transferred into a sterile glass conical flask which was sealed with parafilm. The flasks were then incubated overnight at 37°C in a shaking incubator set at 80 rpm.

Following overnight incubation the flasks were removed, wiped with ethanol and placed in the hood for further processing. The mixture was then disaggregated by repeated pipetting using a 5 ml pipette and the suspension was transferred into a sterile 25 ml universal. A 20 ml syringe and blunt needle were used to further triturate the suspension which was then centrifuged at 770 g for 10 minutes at 20°C.

Meanwhile, type I collagen plates were blocked by incubation with 5 ml of 0.3% (w/v) heat-inactivated BSA in DPBS for 1 hour at 37°C, 5% (v/v) CO₂.

Following the centrifugation step, a plastic Pasteur pipette was used to very carefully pipette off the supernatant. The pellet was then washed twice by re-suspending in 10 ml of sterile DPBS and centrifuging at 770 g for 10 minutes at 20°C. The pellet was then re-suspended in 10 ml R10 and was centrifuged at 308 g for a further 1 minute at 20°C. During this step, the epithelial cells settled to the bottom of the universal whereas the fibroblasts and other stromal cells remained suspended in the supernatant. A plastic Pasteur pipette was rinsed with sterile DPBS and used to collect the settled epithelial cells and transfer them to a sterile 25 ml universal whilst avoiding the collection of too much supernatant. The supernatant was re-centrifuged at 308 g for 1 minute at 20°C and the epithelial cells were again collected and transferred. This step was repeated until all epithelial cells had been collected and they were subsequently washed in 5 ml sterile DPBS by centrifugation at 770 g for 10 minutes at 20°C.
Meanwhile, the remaining fibroblasts and stromal cells were transferred directly along with the R10 media into a T25 tissue culture flask and were cultured at 37°C, 5% (v/v) CO₂.

Following the centrifugation step, a plastic Pasteur pipette was used to carefully remove the supernatant, the cells were re-suspended in 5 ml of pre-warmed trypsin+EDTA and the suspension was incubated for 30 minutes at 37°C in a shaking incubator set at 80 rpm. After the elapsed time, the trypsin was inhibited with 5 ml of R10 and the cells were centrifuged at 577 g for 3 minutes at 20°C. The pellet was then re-suspended in 5 ml R10 and was centrifuged for a further 3 minutes at 577 g at 20°C. Next, the pellet was re-suspended in 3 ml of PEG media and was pipetted repeatedly in order to achieve a single-cell suspension. The suspension was then pipetted over a BD falcon cell strainer above a 50 ml flacon tube and was incubated at 37°C, 5% (v/v) CO₂.

Meanwhile, the cell strainer was inverted over a sterile Petri dish and 3 ml of PEG media was pipetted onto it in order to transfer any remaining tissue onto the dish. A further 5 ml of PEG media was pipetted over the cell strainer and the dish was incubated at 37°C, 5% (v/v) CO₂.

The blocked type I collagen plate was rinsed with 5ml sterile DBS prior to plating out the warmed epithelial cell suspension, which was then incubated for 20 minutes at 37°C, 5% (v/v) CO₂. During this time, the cells expressing high levels of the collagen binding receptor integrin α2β1 bound tightly to the collagen plate whereas those expressing low levels of the receptor remained suspended in the PEG media. The PEG media containing the integrin α2β1 low expressing cells was then collected from the plate and transferred into a sterile 25 ml universal. The plate was subsequently rinsed with 5 ml sterile DPBS which was collected and combined with the media plus integrin α2β1 low-expressing cells. The rinse step was repeated until cells were no longer observed in suspension.

The collected washes plus media with the integrin α2β1 low population was centrifuged at 577g for 3 minutes, the pellet was re-suspended in 3 ml PEG media and the cells were plated onto a small non-blocked collagen plate with 1 ml of the irradiated STO suspension. The plate was incubated at 37°C, 5% (v/v) CO₂.

The integrin α2β1 high-expressing cells were cultured on the collagen plate which was supplemented with 5 ml PEG media plus 2 ml of the irradiated STO suspension and then incubated at 37°C, 5% (v/v) CO₂.
The cells were monitored daily during the week and, with the exception of the weekend, the media was replaced every other day (Monday mornings, Wednesday afternoons and Friday evenings).

2.2.1.2.1 Modifications to the method

The method described was utilised when the ultimate goal was to isolate CD133+ putative prostate cancer stem cells. However, due to the limitations of the method, namely the use of irradiated fibroblasts and the size of the collagen dishes, the method was optimised when cells were cultured/bulked for other areas of interest.

Modifications to the method included the omission of the integrin α2β1-high selection step, the use of collagen coated T25 and T75 tissue culture flasks, the culturing of remaining tissue bits in collagen dishes in PEG media+2% (v/v) FCS, and the omission of irradiated murine fibroblasts for the culture of epithelial cells.

2.2.2 Flow Cytometry and Immunofluorescence

2.2.2.1 Flow Cytometry

During this project, flow cytometry was exploited to identify and quantify different populations of cells expressing a given marker/markers. Antibodies were either used in accordance with manufacturer’s recommendations or were titrated to determine the optimum working concentrations prior to use. Where possible, appropriate positive and negative controls were utilised.

2.2.2.1.1 Extracellular staining protocol

Cells were harvested by trypsinisation, counted using a haemocytometer and 1x 10^6 cells were distributed per flow cytometry tube. The cells were then washed twice in 2 ml flow cytometry buffer (0.5% (w/v) BSA in DPBS) by centrifugation at 400 g for 3 minutes at 4°C. Afterwards, the cells were labelled with primary/conjugated antibody and were incubated in accordance with the manufacturers’ instructions. Following incubation, the cells were washed twice with 2 ml flow cytometry buffer, as previously described, and unconjugated antibodies were incubated with their respective secondary antibodies. Cells were then washed twice more in 2 ml flow cytometry buffer and were re-suspended in 300-400 µl of Isoton and kept on ice prior to flow cytometric analysis using a Beckman Coulter
Gallios flow cytometer. Histograms and dot plots were derived and analysed using the Beckman Coulter Kaluza version 1 software program.

2.2.2.1.2 Intracellular staining protocol

Cells were harvested by trypsinisation, counted using a haemocytometer and 1.5x 10⁶ cells were distributed per flow cytometry tube. To remove cell culture media, 2 ml DPBS was added and the cells were centrifuged for 10 minutes at 300 g. For fixation, cells were resuspended in 1 ml DPBS then 1 ml of 4% (w/v) paraformaldehyde was added to make a final concentration of 2% (w/v) paraformaldehyde and cells were incubated for 10 minutes at 37°C. Following incubation, cells were chilled on ice for 1 minute. The paraformaldehyde was washed off using 2 ml DPBS followed by a centrifugation step of 400 g for 3 minutes. The supernatant was discarded and the cell pellets were resuspended in the tubes and chilled on ice prior to adding 1 ml 90% (v/v) ice-cold methanol and incubating on ice for 30 minutes. The methanol was washed off using 2 ml of cold flow cytometry buffer (0.5% (w/v) BSA in 1x DPBS) followed by a centrifugation step of 400 g for 3 minutes, this step was repeated twice. Cells were resuspended in 100 μl of flow cytometry buffer prior to addition of primary/conjugated antibodies. Cells were incubated with the desired antibody/antibodies for 30 minutes in the dark at 4°C after which time they were washed, as previously, by two centrifugation steps with 2 ml cold flow cytometry buffer. Where applicable, secondary antibodies were applied and incubated as for the primary antibodies. The secondary antibodies were removed using flow cytometry buffer (as previously described) prior to resuspending the cells in 400 μl of Isoton. Flow cytometric analysis was performed using a Beckman Coulter Gallios flow cytometer. Histograms and dot plots were derived using the Beckman Coulter Kaluza version 1 software program.

2.2.2.2 Immunofluorescence

This technique was employed several times throughout the duration of this project to observe the expression of various markers, to identify different cell populations, to determine the purity of isolated cell populations and to observe cell behaviour in response to different treatments. Prior to use, all antibodies were titrated to determine the optimum working concentrations and, where applicable, appropriate positive and negative controls were utilised.
2.2.2.2.1 24 well plate format

In a laminar flow hood, glass coverslips were dipped in 100% methanol, placed into wells of a 24 well plate and allowed to air-dry. Once dry, 0.5 x 10⁵ adherent cells were seeded onto the glass coverslips and the plates were incubated for a minimum of 36 hours at 37°C, 5% (v/v) CO₂. Following incubation, the media was removed and cells were washed once with x1 DPBS. Cells were then fixed with 500 μl 4% (w/v) paraformaldehyde at room temperature for 15 minutes. Next, the paraformaldehyde was removed and the cells were washed twice with 1 ml x1 DPBS. Cells were then blocked and permeabilised using 10% (w/v) BSA in 1x DPBS-0.1% (v/v) TWEEN (blocking solution) for 1 hour at room temperature. Meanwhile, antibodies were prepared by diluting with blocking solution. Following the blocking step, cells were incubated with 200 μl of prepared antibody for 1 hour at room temperature on a rocking platform. To remove unbound primary antibody, the cells were washed 3 times for 10 minutes with 1 ml x1 DPBS-0.1% (v/v) TWEEN prior to incubation with 200 μl of secondary antibody for 1 hour at room temperature on a rocking platform. The plates were wrapped in silver foil to minimise light exposure. The secondary antibody was then removed and the cells were washed 3 times for 10 minutes with 1 ml x1 DPBS-0.1% (v/v) TWEEN. The coverslips were subsequently removed from the wells, inverted and placed on microscope slides with DAPI mounting media. Hence, the cells were face-down on the slides and the nuclei were stained. In order to secure the coverslips, colourless nail varnish was applied around the edge. The slides were then wrapped in foil and transported to an Olympus BX51 fluorescence microscope for imaging.

2.2.2.2.2 96 well fluorescence-compatible plate format

Cells were plated at the desired density (subject to variation depending on the assay). Following completion of the assay, culture media was removed from the wells and cells were washed once with DPBS prior to fixation with 50 μl 4% (w/v) paraformaldehyde for 15 minutes at room temperature. Next, the paraformaldehyde was removed and cells were washed twice with 200 μl x1 DPBS prior to incubation with 50 μl blocking solution for 1 hour at room temperature. Cells were labelled with primary and secondary antibodies and washed as per 24 well plate protocol (volumes were scaled down to 50 μl of antibody solution and 200 μl 1x PBS-0.1% (v/v) TWEEN per well). Following the final wash step, all liquid was removed from the wells and the nuclei were stained with 10 μl of mounting
fluid with DAPI per well. The plate was then wrapped in foil and transported to an Olympus BX51 fluorescence microscope for imaging.

2.2.2.2.3 Immunofluorescent staining of spheres

Cells were cultured at a density of 10,000 cells/well, 4 wells per clone, in ultra low adherent 24 well plates (See section 2.2.6.4.2). The antibody staining protocol was executed as per the 24 well plate format. In order to avoid loss of spheres, washes and blocking solutions were collected in addition, centrifuged in 20 ml universal tubes at 250 g for 10 minutes and collected spheres were stained in parallel. Nuclear staining was achieved by transferring the spheres, resuspended in 50 μl DPBS, onto a glass slide with DAPI mounting media and covering with a large, rectangular coverslip. Prior to imaging, on an Olympus BX51 fluorescence microscope, slides were sealed with colourless nail varnish.

2.2.2.2.4 Immunofluorescent staining of tissue sections

Patient prostate cancer and benign prostatic hyperplasia tissues and tumours from in vivo studies were sectioned onto sialinised slides using a Cryostat and frozen at -80°C. Prior to immunofluorescent staining, the slides were removed, dried and the sections were circled using an ImmEdge hydrophobic barrier pen. Fixing, blocking and staining procedures were executed as per the 24 well plate format, however, volumes were scaled down accordingly and a rocking platform was not utilised. Nuclear staining was achieved by pipetting DAPI mounting media directly onto the sections which were then covered with a large, rectangular coverslip. Prior to imaging, on an Olympus BX51 fluorescence microscope, slides were sealed with colourless nail varnish.

2.2.3 Magnetic Activated Cell Sorting (MACS)

MACS technology has been used throughout the project on a number of different cell populations in attempt to isolate stem cells or cancer stem cells which were present as a very small percentage in a large number of cells. The technique was optimised at the Anthony Nolan Cell Therapy Centre using cord blood to isolate cord blood stem cells according to their expression of the cell surface marker CD133 and the method was then applied when attempting to isolate CD133 expressing cells from prostate cancer cell lines and primary prostate cancer cells.
### 2.2.3.1 MACS of Prostate Cancer Cells from Cell Lines and Primary Cultures

This method was used in an attempt to isolate CD133-positive putative prostate cancer stem cells.

To isolate CD133-positive populations from cell lines, the cells from 10 T175 flasks (approximately 1x10^8 cells; 80% confluency) were harvested by trypsinisation whereas, to isolate CD133-expressing cells from primary cultures, the cells from a maximum of four T75 flasks were harvested by trypsiniation. The cells were then washed in appropriate media by centrifugation at 400 g for 3 minutes, the pellets were re-suspended in appropriate media and the cells were pooled and washed once more. The pooled pellet was re-suspended in 10 ml of MACS buffer and the cells were counted using the trypan blue exclusion method. One million cells were removed from the cell suspension and retained for future analysis as the “mixed fraction”. The remaining cells were spun down and the pellet was labelled with 300 µl MACS buffer, 100 µl FcR block and 100 µl CD133 microbeads. The tube was gently agitated to ensure mixing and the cells were incubated with intermittent agitation for 30 minutes at 4°C in a cold room. After the elapsed time, the cells were re-suspended in 10 ml MACS buffer and were centrifuged at 300 g for 10 minutes. Meanwhile, an LS column was prepared by washing 3 times with 1 ml MACS buffer, ensuring that the column was not dry prior to addition of the cell suspension. After centrifugation, the supernatant was discarded, the cells were re-suspended in 5 ml MACS buffer and the cell suspension was pipetted 1 ml at a time onto a pre-separation filter (placed above the LS column). Once the cell suspension had passed through the column (CD133 negative cells passed through and were captured in a 50 ml falcon tube) the column was washed, 1 ml at a time, with 12 ml MACS buffer. The column was then removed from the magnet, the filter was discarded, 5 ml of MACS buffer was applied directly to the column and the CD133 positive cells were rapidly purged from the column into a 50 ml falcon tube. This step was repeated 3 more times. Hence, the positive fraction was suspended in 20 ml MACS buffer. The positive fraction was centrifuged for 3 minutes at 400 g, the supernatant was discarded and the cells were re-suspended in 500 µl MACS buffer.

In order to increase the purity, the CD133 positive fraction was applied to a second, smaller column. An MS column was prepared by washing 4 times with 500 µl MACS buffer and the positive fraction was applied directly to the column. Once the cell suspension had passed through, the column was washed 8 times with 500 µl MACS buffer.
The MS column was then removed from the magnet and the cells were purged out with 1 ml MACS buffer. This step was repeated 3 more times. Hence, the positive fraction was suspended in 4 ml MACS buffer.

Following isolation the mixed, positive, and negative fractions were analysed immediately by flow cytometry, plated out for immunofluorescence, or plated into 6 well plates and analysed at a later date.

2.2.4 Molecular Biology Techniques

2.2.4.1 RNA Extraction from Cell Lines and Cord Blood Stem Cells

To conduct gene expression analyses, high quality, non-degraded nucleic acid was extracted from several cell types. In order to avoid RNA degradation by RNAses, all RNA extraction procedures and consequent assays were conducted with RNAse free materials in a laminar flow hood.

To extract RNA from cord blood stem cells, 0.5 ml RNA STAT 60 was added directly to the cryovial in which the isolated stem cells had been frozen and the mix was incubated at room temperature for 5 minutes. All reagents were scaled down by half when extracting RNA from cord blood stem cells.

To extract RNA from prostate cell lines cultured in T75 flasks, 1 ml of RNA STAT 60 was pipetted onto the cells and the flask was rotated to ensure complete coverage. A cell scraper was then used to scrape all lysed cells and RNA stat 60 into one corner of the T75 flask. The mix was subsequently pipetted into a 1.5 ml eppendorf tube and was incubated at room temperature for 5 minutes.

Subsequently, 0.2 ml of chloroform was added and the eppendorf was shaken vigorously for 60 seconds. After incubation at room temperature for 3 minutes the eppendorf was centrifuged at 4°C for 10 minutes at 12,000 g. Following centrifugation three layers formed and the clear upper layer was transferred into a sterile 1.5 ml eppendorf. Next, 0.5 ml of isopropanol was added and the eppendorf was repeatedly inverted for 30 seconds to precipitate the RNA. This step was followed by an incubation of 8 minutes at room temperature followed by centrifugation at 4°C for 5 minutes at 12,000 g. The supernatant was then discarded and the white RNA pellet was washed in 1 ml 75% (v/v) ethanol by vortexing. In order to remove the ethanol, the eppendorf was centrifuged at 7,500 g for 5
minutes and the supernatant was discarded. The eppendorf was left open for 10-15 minutes to enable the RNA pellet to dry. Once the ethanol had evaporated, the pellet was re-suspended in 20 μl molecular grade water. Finally, RNA concentration, quantity and quality were evaluated using a NanoDrop UV spectrophotometer at wavelengths of 260 nm and 280 nm.

2.2.4.2 Reverse Transcription

Prior to quantitative RT-PCR, RNA must be converted into cDNA in an enzymatic reaction known as reverse transcription. Firstly, 2 μg of RNA was incubated with 1 μl Oligo DT and 13 μl molecular grade water for 5 minutes at 70°C. Following this step, samples were placed immediately on ice to prevent the formation of secondary structures. To this mixture, the following components were added: 5 μl 5x Buffer, 1 μl dNTPs, 0.7 μl RNAsin RNAse inhibitor, 2.3 μl molecular grade water and 1 μl MLV-Reverse transcriptase. Samples were then incubated in a water bath for 80 minutes at 42°C, after which time the reaction was terminated by a 5 minute heat-step at 95°C. The samples were then pulse-centrifuged prior to storage at 4°C.

2.2.4.3 Quantitative Real-Time PCR

Real-time quantitative RT-PCR was utilised to evaluate the mRNA expression levels of the markers of interest (CD44 and CD133) in prostate cancer cell lines and cord blood stem cells.

Each reaction was prepared on ice in duplicate in 0.2 ml PCR strip tubes. Each tube contained 4.75 μl molecular grade water, 6.25 μl SYBR-green master mix, 0.5 μl forward and 0.5 μl reverse oligonucleotide primers and 0.5 μl of cDNA template. A Rotor-Gene Q real-time PCR detection system was used and GAPDH, HPRT-1 and TBP were used as reference genes to standardise the results.

Primers:

CD44 Forward: 5’-GCCAGAGGCCCAGACAGGCT-3’
CD44 Reverse: 5’-TGGGGCGACAGGTAGAGGCT-3’
CD133 Forward: 5’-TGGCTGGGTGGCCTGGTCAT-3’
CD133 Reverse: 5’-ACGCCTCCGGTGTTGGAGACA-3’
2.2.5 Acquisition of OPCT-1 clones

2.2.5.1 Isolation of Single Cells by Live Cell Laser-Capture Microdissection

During this project laser-capture microdissection was attempted as a means of isolating single E-Cadherin-positive cells for expansion and subsequent investigation of EMT.

1x $10^5$ parental OPCT-1 cells were plated onto a Duplex dish in 2 ml of culture media and incubated overnight at 37°C with 5% (v/v) CO$_2$. Following incubation, the medium was removed, the cells were washed twice with 1 ml of culture media, the primary antibody, diluted in 1 ml of culture media, was applied and the cells were incubated for 1 hour at 37°C, 5% (v/v) CO$_2$. Next, the primary antibody was removed, the cells were washed three times with 1 ml of culture media, the fluorescent conjugated secondary antibody, diluted in 1 ml culture media, was applied and the cells were incubated for 1 hour at 37°C, 5% (v/v) CO$_2$. Following incubation, the secondary antibody was removed and the cells were washed three times with 1 ml of culture media, prior to a 30 minute incubation step with 1 ml culture media at 37°C, 5% (v/v) CO$_2$.

Prior to conducting live cell microdissection, the media was removed and 100 μl of media was applied. The plate was subsequently examined using the Palm laser-capture microdissector, E-Cadherin positive and negative cells were identified, microdissected from the plate and transferred into a 96 well plate with conditioned media. The 96 well plate was incubated at 37°C, 5% (v/v) CO$_2$ and examined daily for cell growth.

2.2.5.2 Cloning of the OPCT-1 Cell Line by Limiting Dilution

This technique was utilised to derive clones with distinct E-Cadherin/vimentin and CD44/CD24 expression profiles from the OPCT-1 cell line.

OPCT-1 cells were harvested by trypsinisation, counted and diluted in culture media to a concentration of 1 cell per 600 μl. This suspension was then plated out in 96 well plates, 200 μl per well, in order to obtain a count of 0.33 cells per well. The plates were examined immediately to ensure that multiple cells were not present in the wells. Plates were then incubated at 37°C, 5% (v/v) CO$_2$ and examined twice a week until single colonies of cells were observed.
2.2.5.3 Bulking, Screening and Selection of the OPCT-1 Clones

This procedure yielded 74 clones. Following ~21 days of culture, the clones were transferred from the 96 well plates to 6 well plates by trypsinisation. Once confluent, they were harvested from the 6 well plates, frozen down for cell culture and re-plated in the 6 well plates. Once confluent, the clones were frozen down a second time, re-plated and screened in duplicate for the expression of E-Cadherin/vimentin and CD44/CD24 by immunofluorescence. The clones were then frozen down a third time prior to selecting suitable clones for further examination. A total of 51 clones were bulked, frozen down and screened by immunofluorescence. Twelve clones were selected for further investigation, these were cultured and screened 3 times by IF (as above) at different passages to ascertain the stability of their phenotypes. From these, 5 clones with distinct E-Cadherin/vimentin expression profiles were selected and interrogated for the purpose of this study.

2.2.5.4 Cell Sorting – Re-cloning of OPCT-1 Clones

In order to re-confirm that a single OPCT-1 cell was capable of generating both epithelial and mesenchymal cell types, it was necessary to re-clone the OPCT-1 clones using a MoFlo™ XDP High-Speed Cell Sorter.

Single cell suspensions of the 5 OPCT-1 clones of interest were sorted at a density of one cell per well (32 wells per clone) into fluorescence compatible 96 well plates using a MoFlo™ XDP High-speed cell sorter. The plates were incubated at 37°C, 5% (v/v) CO₂ for 14 days after which time the cells were fixed with 4% (w/v) paraformaldehyde for 15 minutes at 4°C, stained with anti-E-Cadherin and anti-vimentin (See Section 2.2.2.2.2) and imaged using an Olympus BX51 fluorescence microscope. This assay was conducted in three separate experiments. The mean colony forming efficiency of each of the clones was calculated by dividing the average number of colonies which grew per clone by the number of wells seeded (32) and multiplying by 100.
2.2.6  Characterisation of OPCT-1 clones

The following assays were executed in order to characterise the OPCT-1 clones and investigate a possible link between the cancer stem cell phenotype and epithelial to mesenchymal transition. In parallel to each assay, the phenotype of the clones was monitored by immunofluorescence.

2.2.6.1  Investigating the Protein Expression of Stem and EMT-Associated Markers in OPCT-1 Clones

In order to compare the protein expression profiles of the OPCT-1 clones, both Western blotting and immunofluorescence were conducted using a panel of several EMT and stem cell-associated markers.

2.2.6.1.1  Preparation of cell lysates

Following culture in standard conditions, 70-80% confluent cells growing in T175 tissue culture flasks were washed twice with sterile DPBS and scraped in the residual DPBS using a cell scraper. The cells+DPBS were transferred into an eppendorf tube and centrifuged at 10,000 g for 10 minutes. Immediately after centrifugation, the supernatant was discarded, the pellets were lysed in total cell lysis buffer (volumes depended on the size of the pellet) and the lysate was heated in a thermoblock at 99°C for 10 minutes. Lysates were either stored at -20°C for future use or the protein concentration was immediately quantified using the BCA assay.

2.2.6.1.2  BCA Protein Assay

This assay was conducted in order to calculate the concentration of total protein in the cell lysates and was executed in accordance with the manufacturers’ instructions. In brief, triplicate standards were prepared in a flat bottom 96 well plate from a stock BSA solution (1 mg/ml) of the range 0, 5, 10, 15, 20, 25 μg and the volumes were adjusted to 25 μl with total cell lysis buffer. Cell lysates were pipetted in triplicate wells, 2.5 μl/well and the total volume was adjusted to 25 μl with total cell lysis buffer. Next, solution A and solution B were prepared in a 1:50 dilution, in accordance with the manufacturer’s instructions, and 200 μl was distributed per well. The plate was then covered with a plastic seal and incubated at 37°C, 5% (v/v) CO₂ for 30 minutes. After the elapsed time, the plate was read using a Tecan Ultra microtiter plate reader at a wavelength of 560 nm. Using Excel, a
standard curve was plotted from the BSA standard absorbance data and this was used to quantify the concentration of protein in the lysates.

2.2.6.1.3 Western Blot Analysis of Protein

2.2.6.1.3.1 SDS PAGE

Prior to immunoblotting, SDS PAGE was conducted in order to separate the proteins in a sample.

SDS gels were prepared and immersed in a tank containing 1x tris-glycine-SDS running buffer. Lysates were prepared with sample reducing buffer at a ratio of 3:1 (lysate: reducing buffer) by heating on a thermoblock at 95°C for five minutes. Next, sample containing 20 or 30 μg of protein (depending on the experiment) was loaded into the wells of the SDS gels alongside a molecular weight marker. Once loaded, 80V were applied to the gels for 10 minutes to allow the proteins to pass through the stacking gel, this was increased to 150V to allow the proteins to separate in the resolving gel.

2.2.6.1.3.2 Wet Transfer

Once the proteins had run through the gel (~75 minutes), they were transferred onto a nitrocellulose membrane via “wet transfer”. Briefly, a “sandwich” was prepared using a gel frame, a sponge soaked in transfer buffer, two pieces of filter paper soaked in transfer buffer, the SDS gel, a nitrocellulose membrane, two additional pieces of transfer buffer-soaked filter paper and one more transfer buffer-soaked sponge. This was sealed with another gel frame and inserted into a transfer tank filled with ice cold transfer buffer. Proteins were transferred overnight for 16 hours at 30V or alternatively, for one hour at 100V.

2.2.6.1.3.3 Copper staining

In order to image the protein bands, hence confirm a successful transfer process and equal protein loading, nitrocellulose membranes were stained for 10 minutes in Copper phthalocyanine-3,4',4″,4‴-tetrasulfonic acid tetrasodium salt and imaged using a darkbox. The membranes were then cut to allow for probing with different antibodies. Prior to immunoprobing, the membranes were de-stained in DPBS with two drops of 10 M sodium hydroxide and then blocked with 3% (w/v) Marvel milk solution for one hour at room temperature on a rocking platform.
2.2.6.1.3.4 Immunoprobing

Prior to obtaining the final results, all antibodies were titrated using a mixed cell lysate in order to determine optimum working dilutions.

Cut nitrocellulose membranes were incubated with the corresponding primary antibody diluted in 3% (w/v) Marvel milk solution on a rocking platform over night at 4°C. The following day, the membranes were washed three times for 10 minutes in DPBS-0.1% (v/v) TWEEN prior to adding the corresponding HRP-linked secondary antibody diluted in 3% (w/v) Marvel milk solution. A streptavidin-HRP secondary antibody was used to stain the molecular weight marker present on each membrane. Following one hour incubation at room temperature on a rocking platform, the membranes were washed as previously described. The membranes were developed using ECL reagent which was applied for one minute prior to exposure (exposure times varied depending on the abundance of the protein) and development using a CCD camera.

2.2.6.2 Proliferation Assays

The proliferation of the OPCT-1 clones was investigated using the [3H]-Thymidine incorporation assay, the ViaLight® Plus kit and the MTT assay.

2.2.6.2.1 [3H]-Thymidine Incorporation Assay

This assay assesses proliferation based on the incorporation of [3H]-Thymidine into the cells’ DNA during replication. Prior to obtaining the final results, this method was optimised in terms of plate format, cell density, incubation periods and harvesting method. Briefly, 10,000 cells were plated in quadruplicate wells of a 96 well plate and incubated for 24 hours at 37°C, 5% (v/v) CO₂. [3H]-Thymidine was added to the cells with a final concentration of 0.037 MBq/ml and incubated at 37°C with 5% (v/v) CO₂ for 19.5 hours. A Filtermate Harvester was utilised to transfer the media from the cells grown in the 96 well plate to a 96 well Unifilter plate. The cells were then incubated for 15 minutes with 30 μl of trypsin prior to harvesting onto the Unifilter plate using the Filtermate Harvester and the plate was then left for 2 hours at room temperature to dry. Once dry, the Unifilter plate was overlaid with 40 μl of Microscint-O scintillation fluid and analysed using an NXT Top-Count microplate scintillation counter to detect radioactivity in the form of “counts per minute” (cpm) for each well. This assay was conducted in three separate experiments. Data obtained were analysed using GraphPad Prism 5 software.
2.2.6.2 ViaLight® Plus Proliferation Assay

The ViaLight® Plus proliferation assay detects cellular proliferation by determination of ATP levels using bioluminescence based on the luciferin-luciferase reaction. Prior to obtaining the final results, this method was optimised in terms of cell density and incubation periods.

This assay was conducted as per the manufacturer’s recommendations. In brief, 10,000 cells in 100 µl of culture media were plated in quadruplicate wells of a 96 well plate and incubated for 24 hours at 37°C, 5% (v/v) CO₂. Following incubation, cells and reagents were brought to room temperature for 15 minutes prior to a 10 minute cell lysis step with 50 µl Cell Lysis Reagent. As a control, 100 µl of media/lysis buffer (1:1) was transferred to 4 wells of a white walled luminometer plate. After cell lysis, 100 µl of cell lysate was transferred to the luminometer plate, 100 µl of the reconstituted ViaLight® Plus reagent (AMR PLUS) was added to each appropriate well and the plate was incubated for 2 minutes at room temperature. The plate was read in a microplate luminometer which took one second integrated readings expressed as relative light units (RLUs). This assay was conducted in three separate experiments. Data obtained were analysed using GraphPad Prism 5 software.

2.2.6.2.3 MTT Proliferation Assay

This assay measures cell proliferation based on the reduction of the yellow tetrazolium salt, 3, [4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), to an insoluble purple formazan dye by mitochondrial enzymes associated with metabolic activity. This assay was optimised previously and was conducted as per recommendation.

Cells were plated at a density of 25,000 per well in 1 ml media in triplicate wells of a 24 well plate and incubated for 24 hours at 37°C, 5% (v/v) CO₂. The reconstituted MTT reagent (5mg/ml) was brought to room temperature and 50 µl was added per well, prior to incubation for 30 minutes at 37°C, 5% (v/v) CO₂. Following incubation, the media was removed from the wells and 500 µl DMSO was added per well and pipetted up and down to solubilise the formazan dye. As a control, 200 µl of DMSO was transferred to 6 wells of a 96 well microtiter plate. Next, 200 µl of DMSO/formazan dye was transferred in duplicate, to the 96 well microtiter plate hence, 6 wells per cell line. The absorbance in each well was read using a Tecan microtiter plate reader at a wavelength of 570 nm. This
assay was conducted in three separate experiments. Data obtained were analysed using GraphPad Prism 5 software.

2.2.6.3 Drug Assays

2.2.6.3.1 Determination of the Docetaxel IC50 for Parental OPCT-1 using the [3H]-Thymidine Incorporation Assay

This assay was executed in order to determine the IC50 dose of docetaxel on parental OPCT-1 for use in drug sensitivity studies on the OPCT-1 clones. Prior to obtaining the final result, this method was optimised in terms of cell density, volume of drug media, addition of [3H]-Thymidine, incubation periods and drug concentration range.

Briefly, 10,000 OPCT-1 cells were plated in 32 wells of a 96 well plate and incubated for approximately 20 hours at 37°C, 5% (v/v) CO₂. Following incubation, the media was removed and replaced with 200 μl control media (media only) and docetaxel-laced media of the range 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1000 nM. The plate was then incubated for 48 hours at 37°C, 5% (v/v) CO₂. Following incubation, [3H]-Thymidine was added to the cells with a final concentration of 0.037 MBq/ml and incubated at 37°C with 5% (v/v) CO₂ for 19.5 hours. As previously, a Filtermate Harvester was utilised to transfer the media from the cells grown in the 96 well plate to a 96 well Unifilter plate. The cells were then incubated for 15 minutes with 30 μl of trypsin prior to harvesting onto the Unifilter plate using the Filtermate Harvester and the plate was left for 2 hours at room temperature to dry. Once dry, the Unifilter plate was overlaid with 40 μl of Microscint-O scintillation fluid and analysed using an NXT Top-Count microplate scintillation counter to detect radioactivity in the form of “counts per minute” (cpm) for each well. This assay was conducted in five separate experiments, in quadruplicate. The data obtained were used to plot a dose response curve and ascertain the IC50 dose using GraphPad Prism 5 software. Using this assay, the docetaxel IC50 for parental OPCT-1 was determined as 5.617 nM.

2.2.6.3.2 Determination of the Docetaxel IC50 on Parental OPCT-1 using the ViaLight® Plus Proliferation Assay

In addition to the [3H]-Thymidine Incorporation Assay, this assay was performed in order to determine the IC50 dose of docetaxel on parental OPCT-1 for use in drug sensitivity studies on the OPCT-1 clones. Prior to obtaining the final results, this method was optimised in terms of cell density, incubation periods and drug concentration range.
In Brief, 10,000 OPCT-1 cells were plated in 32 wells of a 96 well plate and incubated for approximately 20 hours at 37°C, 5% (v/v) CO₂. Following incubation, the media was removed and replaced with 100 μl control media (media only) and docetaxel-laced media of the range 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1000 nM. The plate was then incubated for 48 hours at 37°C, 5% (v/v) CO₂. Following incubation, cells and reagents were brought to room temperature for 15 minutes prior to a 10 minute cell lysis step with 50 μl Cell Lysis Reagent. As a control, 100 μl of media/lysis buffer (1:1) was transferred to 4 wells of a white walled luminometer plate. After cell lysis, 100 μl of cell lysate was transferred to the luminometer plate, 100 μl of the reconstituted ViaLight® Plus reagent (AMR PLUS) was added to each appropriate well and the plate was incubated for 2 minutes at room temperature. The plate was read in a luminometer which took 1 second integrated readings expressed as relative light units (RLUs). This assay was conducted in three separate experiments, in quadruplicate. The data obtained were used to plot a dose response curve and ascertain the IC50 dose using GraphPad Prism 5 software. Using this assay, the docetaxel IC50 for parental OPCT-1 was determined as 11.15 nM.

2.2.6.3.3 Thymidine Clone IC50 Assay

After ascertaining the IC50 concentration of docetaxel on parental OPCT-1, this assay was performed in order to compare the sensitivity of the clones to docetaxel.

Briefly, 10,000 cells were plated in 12 wells of a 96 well plate per clone and incubated for approximately 20 hours at 37°C, 5% (v/v) CO₂. Following incubation, the media from 4 wells per clone was removed and replaced with 200 μl control media (media only), the media from 4 wells per clone was removed and replaced with 200 μl of docetaxel-laced media at 5.5 nM (the IC50) and the media from 4 wells per clone was removed and replaced with 200 μl of docetaxel-laced media at 11 nM (double the IC50). The plate was then incubated for 48 hours at 37°C, 5% (v/v) CO₂. Following incubation, [3H]-Thymidine was added to the cells with a final concentration of 0.037 MBq/ml and incubated at 37°C with 5% (v/v) CO₂ for 19.5 hours. As previously, a Filtermate Harvester was utilised to transfer the media from the cells grown in the 96 well plate to a 96 well Unifilter plate. The cells were then incubated for 15 minutes with 30 μl of trypsin prior to harvesting onto the Unifilter plate using the Filtermate Harvester. Once dry, the Unifilter plate was overlaid with 40 μl of Microscint-O scintillation fluid and analysed using an NXT Top-Count microplate scintillation counter to detect radioactivity in the form of
“counts per minute” (cpm) for each well. This assay was conducted in five separate experiments, in quadruplicate. STATISTICA software was used to analyse the data obtained by means of a Factorial ANOVA. The percentage inhibition of proliferation was calculated by dividing the difference between the values for control and test by the test values and multiplying by 100.

2.2.6.4 Clonogenic, Sphere-forming and Self-renewal Assays

2.2.6.4.1 Clonogenic Assay

This assay was conducted in order to assess and compare the clonogenicity of the OPCT-1 clones and ascertain whether the clones with an EMT phenotype were more clonogenic.

Single cell suspensions were plated at the clonal density of 125 cells per well of a 6 well plate, 2 wells per clone. Following 10 days in culture, the colonies were fixed with 4% (w/v) paraformaldehyde for 15 minutes at 4°C and stained with crystal violet solution (0.5% (w/v) crystal violet in 70% (v/v) ethanol) for 15 minutes at room temperature, after which, the colonies were washed with DPBS and allowed to dry prior to counting under a light microscope. Colonies with fewer than 32 cells were excluded from the counts. The plates were then photographed. This assay was conducted in duplicate wells in three separate experiments. Data collected were analysed using GraphPad Prism 5 software.

2.2.6.4.2 Sphere-Forming Assay

This assay was conducted in order to assess and compare the sphere-forming ability of the OPCT-1 clones and ascertain whether the clones with an EMT phenotype possessed enhanced sphere-forming capabilities. Prior to obtaining final results, this assay was optimised in terms of media composition, plate format, cell density and duration of culture.

Single cell suspensions were plated in triplicate wells of an ultra-low adherent 24 well plate at a density of 10,000 cells per well and were cultured in normal media (KSFM 2% (v/v) FCS) for 12 days at 37°C, 5% (v/v) CO₂. Spheres were observed microscopically and counted by two individuals; an average of the counts was taken. This assay was conducted in triplicate wells in three separate experiments. Data collected were analysed using GraphPad Prism 5 software.
2.2.6.3 Self-Renewal Assay

This assay was conducted in order to assess and compare the self-renewal capabilities of the OPCT-1 clones and ascertain whether the clones with an EMT phenotype possessed enhanced self-renewal abilities.

As with the sphere-forming assay, single cell suspensions were plated in triplicate wells of an ultra-low adherent 24 well plate at a density of 10,000 cells per well and were cultured in 1 ml of normal media (KSFM 2% (v/v) FCS) for 12 days at 37°C, 5% (v/v) CO₂, after which time the spheres were observed microscopically and counted by two individuals. Next, the spheres and culture media were collected from 2 wells per clone (handled separately) and centrifuged for 3 minutes at 300 g. The supernatant was discarded and the spheres were re-suspended in 300 μl trypsin and versene (T&V) prior to incubation at 37°C for 30 minutes. Following incubation, the spheres were further dissociated by persistent pipetting and were subsequently sorted at a density of 750 cells per well of an ultra low adherent 24 well plate with 750 μl KSFM 2% (v/v) FCS+pen/strep using a MoFlo™ XDP High-speed cell sorter. Cells were cultured for 12 days at 37°C, 5% (v/v) CO₂ after which time, the wells were examined microscopically for the presence of secondary sphere formation. Spheres were counted by two individuals and an average of the counts was taken. This assay was conducted in triplicate wells in three separate experiments. Data collected were analysed using GraphPad Prism 5 software.

2.2.6.5 Aldefluor™ Assay

Aldehyde dehydrogenase 1 (ALDH1) has been shown to be essential for stem cell survival and early differentiation and has been utilised to identify haematopoietic, mammary and neural stem cells. ALDH1 activity has also been exploited as a potential marker for CSCs and ALDH1hi populations have been shown to be highly tumourigenic. Therefore, the Adefluor™ assay was conducted in this study in order to ascertain whether the clones with an EMT phenotype possessed a larger subpopulation of cells with enhanced ALDH1 activity.

Prior to obtaining final results, this assay was optimised in terms of incubation periods, cell numbers and volume of reagents used.

Briefly, cells were harvested and counted and a suspension of 1x10⁶ cells per clone was made up to 1 ml with Aldefluor™ Assay Buffer and split equally between two flow
cytometry tubes. To inhibit ALDH1 activity in the control tubes, 2.5 μl of Aldefluor™ DEAB Reagent was added and incubated at room temperature for 10 minutes. Next, 2.5 μl of activated Aldefluor™ Reagent was added to each tube prior to incubation 37°C, 5% (v/v) CO₂ for 45 minutes. After the elapsed time, cells were centrifuged at 400 g for 3 minutes, to remove excess substrate. Cells were subsequently resuspended, on ice, in 500 μl of cold Aldefluor™ Assay Buffer. In order to avoid loss of staining, as a result of efflux from the cells, cells were analysed immediately using a Beckman Coulter Gallios flow cytometer and cells awaiting analysis were kept covered, on ice at all times. Furthermore, the order in which the cells were analysed was changed in each repeat of the experiment. This assay was conducted in four separate experiments. Histograms and dot plots were derived using the Beckman Coulter Kaluza version 1 software program. The percentage of ALDH1hi cells was determined by gating around a density plot of control cells (treated with DEAB reagent), applying the same gate to the test cells and subtracting the number of cells within the control gate from the number of cells within the test gate. Data obtained were analysed using GraphPad Prism 5 software and the mean percentage of ALDH1hi cells present in each clone, compared with parental OPCT-1, was calculated.

2.2.6.6 Invasion and Migration Assays

2.2.6.6.1 Scratch Assay

This assay was performed in order to observe the possible migration of mesenchymal (vimentin-positive) cells into an artificial wound. Prior to obtaining final results, this assay was optimised in terms of media composition, cell density, scratching technique and duration.

Fluorescence-compatible 96 well plates were coated during a 1 hour incubation with 30 μl poly-L-lysine per well. The unbound poly-L-lysine was subsequently removed from each well prior to seeding cells in quadruplicate wells at the high density of 50,000 cells per well. Twenty four hours later, 200 μl pipette tips coated with poly-L-lysine were employed to create a scratch in the confluent monolayer, in the centre of each well. The cells were washed twice with sterile DPBS prior to being cultured in serum free KSFM media for 12 hours at 37°C, 5% (v/v) CO₂. After the elapsed time, cells were fixed with 4% (w/v) paraformaldehyde for 15 minutes at 4°C, stained with anti-E-Cadherin and anti-vimentin (See Section 2.2.2.2.2) and observed using an Olympus BX51 fluorescence microscope. This assay was conducted in four separate experiments.
In Vitro Matrigel\textsuperscript{TM} Invasion Assay

This assay was conducted in order to compare the invasiveness of the OPCT-1 clones and observe a possible connection between EMT and invasion. For this purpose, 24 well Biocoat Matrigel\textsuperscript{TM} Invasion Chambers containing BD Falcon Cell Culture Inserts with 8 \( \mu \)m pore-size PET membranes that had been treated with Matrigel\textsuperscript{TM} Matrix were used. Prior to obtaining final results, this assay was optimised in terms of cell density, media composition and method of enumeration.

Cells were serum-starved for 24 hours prior to conducting the assay. Two hours prior to plating the cells, Matrigel\textsuperscript{TM}-containing inserts were hydrated using 500 \( \mu \)l serum-free culture medium and incubated 37\(^\circ\)C, 5% (v/v) CO\(_2\). Meanwhile, cells were counted and re-suspended in 2.5 ml serum free KSFM. After hydration, the media was removed from the Matrigel\textsuperscript{TM} inserts and the Matrigel\textsuperscript{TM} and control inserts were transferred into a sterile 24 well companion plate. Next, 750 \( \mu \)l of chemoattractant, KSFM 10% (v/v) FCS, was pipetted into each well, avoiding the inserts. The cells were then plated carefully into the Matrigel\textsuperscript{TM} and control inserts at a density of 1.5x10\(^6\) cells per insert (500 \( \mu \)l cell suspension) and were incubated at 37\(^\circ\)C, 5% (v/v) CO\(_2\) for 22 hours. After the elapsed time, tweezers were utilised to remove the inserts and pour off the media. In order to remove Matrigel\textsuperscript{TM} matrix and non-invading cells, the inside of each insert was scrubbed with a DPBS-soaked cotton bud. After scrubbing, the cells were fixed by placing each insert into a companion plate with 750 \( \mu \)l ethanol and incubating on ice for 15 minutes. Meanwhile, two 24 well wash plates were prepared with 750 \( \mu \)l DPBS per well. After the elapsed time, excess ethanol was poured from the inserts prior to placing them into the first wash plate and incubating for two minutes. Next, the wash step was repeated by transferring the inserts from the second wash plate and incubating for a further two minutes. Meanwhile, a 0.1 mg/ml solution of propidium iodide (PI) was prepared, by diluting the stock solution (1 mg/ml) with DPBS and 750 \( \mu \)l of PI solution was pipetted into each well of a new companion plate. The inserts were then transferred from the second wash plate into the companion plate with PI, wrapped in aluminium foil and incubated at 37\(^\circ\)C, 5% (v/v) CO\(_2\) for 20 minutes. Following incubation, the inserts were washed, as previously, air dried and transferred into a new companion plate prior to scanning and imaging using a C.T.L ELISPOT plate reader. The images were analysed using ImmunoSpot\textsuperscript{®} software. Percentage invasion was calculated by dividing the cell counts for the Matrigel\textsuperscript{TM} inserts by the counts for the control inserts and multiplying by 100. This assay was conducted in
three separate experiments. Data were analysed using Microsoft Excel and GraphPad Prism 5 software.

2.2.6.7 Response to Matrigel™ Assay

Prior to conducting *in vivo* studies with Matrigel™, it was necessary to ascertain whether the OPCT-1 clones would maintain a stable phenotype during incubation with Matrigel™, which contains known inducers of EMT, including TGF-β.

Glass coverslips were plated into 24 well plates (See Section 2.2.2.2.1) and were covered with 100 µl Matrigel™: serum free KSFM media (1:6). Cells were plated onto the Matrigel™ at a density of 10,000 cells per well, in quadruplicate wells and were cultured at 37°C, 5% (v/v) CO₂ for either 72 hours or 7 days. After the elapsed time, cells were fixed with 4% (w/v) paraformaldehyde for 15 minutes at 4°C, stained with anti-E-Cadherin and anti-vimentin and observed using an Olympus BX51 fluorescence microscope.

2.2.6.8 *In vivo* Tumourigenesis

Male nude athymic mice were used as a model to investigate an association between EMT and tumourigenesis. Four OPCT-1 clones of interest, exhibiting distinct E-Cadherin and vimentin expression profiles; P5B3, P6D4, P2B9 and P4B6, plus parental OPCT-1 were injected and monitored over a period of 52 days.

In brief, cells were injected at a density of 2.5x10⁶ with 100 µl 1:6 serum free media: Matrigel™, sub-cutaneously into the right flank of male athymic nude mice, 6 mice per cell line. Tumour development was observed twice weekly using calliper measurements until one of the tumours had reached a diameter of 1 cm, after which time the experiment was terminated and the mice were euthanised. Tumours were then extracted, mounted on cork boards with OCT, snap frozen in liquid nitrogen-cooled 2-butanol and stored at -80°C, prior to cryostat sectioning.

2.2.6.9 Statistical Analysis

Unless stated, all data are presented as mean ± standard error of the mean (SEM). Due to the fact that fewer than 12 replicates were performed in most of the assays conducted throughout this study, most of the data generated did not follow a normal (Gaussian) distribution. Therefore, unless stated, non-parametric tests were utilised to analyse the data.
Data are presented as overall significance between the clones and parental OPCT-1 in a given test represented, in some figures, by asterisks with the significance levels $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***)

When investigating overall significance between the clones and parental OPCT-1, unless otherwise stated, non-parametric testing was performed by means of a Friedman test, where the same number of replicates was used, or a Kruskal-Wallis test, when the number of replicates varied. Significant differences between the clones and parental OPCT-1 were investigated using the Dunn's Multiple Comparison (Post-Hoc) test.
Chapter 3:

Attempted Identification and Isolation of Putative Prostate Cancer Stem Cells

3.1 Introduction

3.1.1 Stem Cells in Prostate Cancer

The stem cell model for prostate epithelia was established by Isaacs and Coffey (1989) following a series of androgen cycling experiments in a rodent model. In demonstrating that the prostate, an androgen-dependent organ, could completely regenerate following castration once androgen levels were restored, they proposed that androgen independent stem cells yield a population of androgen-responsive cells which, in turn, give rise to the more differentiated androgen-dependent secretory luminal cells (Isaacs and Coffey, 1989). This model for the hierarchy of the prostate gland is now widely accepted and prostate stem cells have since been isolated and investigated (Richardson et al., 2004).

In 2005 Collins et al., isolated “tumourigenic prostate cancer stem cells” using the same markers they had previously established for normal prostate tissue stem cells (Richardson et al., 2004; Collins et al., 2005). The marker profile identified for prostate cancer stem cells was integrin $\alpha_2\beta_1$ high/CD44+/CD133+.

Integrin $\alpha_2\beta_1$ (also referred to as VLA-2) is an extracellular matrix protein receptor for laminin, collagen, fibronectin and E-Cadherin. Among other functions, it is known to play a role in the generation and organisation of extracellular matrix proteins.

CD44, a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion, migration and lymphocyte activation, has been implicated as a cancer stem cell marker for several other cancers including breast (Sheridan et al., 2006), head and neck (Chen et al., 2010) and colorectal (Du et al., 2008). Since CD44 is known to play a role in supporting cell survival it is thought to be pro-oncogenic by nature (Naor et al., 2008). According to
the cancer stem cell theory, secondary tumour formation (metastasis) is attributed to the migration and seeding of cancer stem cells at the secondary site. Since CD44 has been shown to play a role in both migration and adhesion, it may be a critical molecule for the cancer cells which express it. Furthermore, the expression of several CD44 proteins has been shown to correlate with aggressive stages of a variety of human cancers (Orian-Rousseau, 2010).

CD133 has also been implicated as a marker for putative cancer stem cells in several other cancers including brain (Uchida et al., 2000; Singh et al., 2003), liver (Ma et al., 2007; Schmelzer et al., 2007), melanoma (Rappa et al., 2008), colorectal (Ricci-Vitiani et al., 2007) and lung (Eramo et al., 2007). To date, the function of this molecule remains to be elucidated. Although very little is known about the role of CD133, since this molecule is often also expressed by the normal tissue stem cells corresponding to the tissue from which the cancer stem cells are identified (See Table 1.1), it provides compelling evidence for the theory that cancer stem cells are derived from transformed adult tissue stem cells.

In 2005, Collins and colleagues demonstrated that cells with the marker profile integrin $\alpha_2\beta_1^{\text{high}}$/CD44$^+$/CD133$^+$ possessed significant capacity for self-renewal, differentiation and invasiveness in vitro, all of which have been attributed to cancer stem cells. Such cells may be responsible for both the initiation and progression of prostate cancer i.e. relapse and metastatic spread despite androgen ablation or other treatments. Therefore, these markers can be exploited to isolate putative prostate cancer stem cells.

As the markers for prostate cancer stem cells had already been reported and had been previously exploited to isolate these cells from a mixed population, this work endeavoured to reproduce the method in order to isolate and further characterise prostate cancer stem cells from both established prostate cancer cell lines and cultured primary prostate cancer cells.
3.2 Results

The prostate cancer cell lines PC3 and DU145 were selected for the preliminary studies because these, well characterised, cell lines were derived from metastatic lesions, as such; are androgen dependent and have been shown to be highly tumourigenic (Coso and Williams, 2009, Malhi et al., unpublished data). Prostate cancer stem cells are believed to contribute significantly to tumourigenesis, therefore, it was hypothesised that these cell lines may provide a suitable model from which to identify, isolate and characterise putative prostate cancer stem cells. Furthermore, in 2007, Wei and colleagues identified a population of “cancer stem-like cells” in the DU145 cell line by exploiting the markers identified by Collins et al., 2005 (Collins et al., 2005; Wei et al., 2007).

Training undertaken with Professor Norman Maitland and his group at The University of York enabled the culture of primary prostate cancer cells from patient samples which were subsequently exploited for the attempted isolation of putative prostate cancer stem cells.

Therefore, this work attempted to reproduce and advance the method reported in order to resolve some of the additional questions regarding the prostate cancer stem cell phenotype.

3.2.1 Analysis of CD44 and CD133 Expression in Prostate Cancer Cell Lines by Quantitative Real-Time PCR

Prior to conducting studies on the expression of PCSC-associated markers at the protein level, quantitative real-time PCR was performed in an attempt to detect CD44 and CD133 in DU145 and PC3 cells at the mRNA level.

To that end, RNA from two metastatic prostate cancer cell lines; PC3 and DU145 and cord blood stem cells (CBSCs), which were used as a positive control, was extracted and reverse transcribed. Forward and reverse primers were designed for CD44 and CD133 and exploited for qRT-PCR. Figures 3.1 and 3.2 demonstrate that CD133 and CD44 were both detected in PC3 and DU145 cells at the mRNA level.
Figure 3.1: Relative expression of CD44 in PC3 and DU145 cells determined by real-time qPCR using forward and reverse primers designed for CD44. (n=3). Results were analysed using the comparative CT method. The housekeeping genes; GAPDH, TBP and HPRT-1 were used for CT value normalisation and cord blood stem cells were used to compare the expression of CD44 in the prostate cancer cell lines.

Figure 3.2: Relative expression of CD133 in PC3 and DU145 cells determined by real-time qPCR using forward and reverse primers designed for CD133. (n=3). Results were analysed using the comparative CT method. The housekeeping genes; GAPDH, TBP and HPRT-1 were used for CT value normalisation and cord blood stem cells were used to compare the expression of CD133 in the prostate cancer cell lines.
Both PC3 and DU145 cells expressed CD44 at the mRNA level, with DU145 expressing higher levels than PC3. However, these cell lines expressed lower levels of CD44 mRNA than cord blood stem cells (Figure 3.1).

Quantitative real-time PCR determined that both PC3 and DU145 cells expressed very low levels of CD133 at the mRNA level, with DU145 expressing higher levels than PC3 (Figure 3.2). As anticipated, both cell lines expressed much lower levels of CD133 mRNA than cord blood stem cells. This was as expected because CD133-positive putative PCSCs have been reported to represent a very small population among a mixed population of predominantly differentiated cells (Collins et al., 2005; Wei et al., 2007). As such, both DU145 and PC3 cells, which had not been enriched for CD133-positive cells, were expected to express lower levels of CD133 mRNA than the purified population of CD133-positive cord blood stem cells.

The detection of the PCSC-associated antigens; CD44 and CD133 in PC3 and DU145 cells at the mRNA level provided justification for further investigation of these markers at the protein level.

3.2.2. Analysis of CD44 and CD133 Expression in Prostate Cancer Cell Lines by Flow Cytometry

To investigate the expression of CD44 and CD133 on the surface of PC3 and DU145 cells and quantify the number of cells expressing these proteins, single-colour flow cytometry was performed, using conjugated antibodies directed against CD44 and CD133. Appropriate isotype controls were used to define the gated regions and the cells which fell into these regions during the test run were considered positive for the expression of the given marker with which the isotype corresponded.

Flow cytometric analysis was initially performed using one antibody per tube. However, it is important to note that putative prostate cancer stem cells have been shown to express both CD44 and CD133 hence, in order to identify PCSCs it would be necessary to conduct analysis on cells labelled with both antibodies. Nonetheless, prior to conducting dual-staining it was necessary to identify if these proteins were expressed by PC3 and DU145 cells.
Figure 3.3: Demonstrating sample flow cytometric data from which the percentage of CD44-positive cells present in the PC3 cell line was determined. Flow cytometry was performed using a FITC-conjugated isotype control to gate the cells and FITC-conjugated anti-CD44 antibody to identify the population of cells expressing CD44. A histogram overlay of isotype versus test is also shown. The positive population was defined as cells present in gate D (CD44-FITC) and was calculated by subtracting the percentage of cells in gate C (iso-FITC) from the percentage in gate D (CD44-FITC). A total of 10,000 events were collected and analysed per sample. (n=3). Representative data.

Figure 3.4: Demonstrating sample flow cytometric data from which the percentage of CD133-positive cells present in the PC3 cell line was determined. Flow cytometry was performed using an APC-conjugated isotype control to gate the cells and an APC-conjugated anti-CD133 antibody to identify the population of cells expressing CD133. A histogram overlay of isotype versus test is also shown. The positive population was defined as cells present in gate B (CD133-APC) and was calculated by subtracting the percentage of cells present in gate A (iso-APC) from the percentage in gate B (CD133-APC). A total of 10,000 events were collected and analysed per sample. (n=2). Representative data.
The mean percentages of CD44 and CD133 positive cells present in the PC3 cell line were calculated from the flow cytometric analyses and are presented in Table 3.1.

Table 3.1 Demonstrating the mean percentage of CD44 and CD133 positive cells in the PC3 cell line as determined by flow cytometry

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mean % Positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>99.52</td>
</tr>
<tr>
<td>CD133</td>
<td>0</td>
</tr>
</tbody>
</table>

Flow cytometric analysis determined that almost all PC3 cells analysed (99.52%) expressed CD44. However, none of the cells were found to express CD133. Therefore, despite the fact that the majority of PC3 cells expressed one of the reported markers of prostate cancer stem cells, a putative cancer stem cell population (as defined by Collins et al., 2005) was not identified in these cells, using this technique.

Table 3.2 reveals that similar results were observed with DU145 cells, the majority of which (94.08%) expressed CD44 however no cells expressing CD133 were detected by flow cytometry (Figures 3.5 and 3.6).

Table 3.2 Demonstrating the mean percentage of CD44 and CD133 positive cells in the DU145 cell line as determined by flow cytometry

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mean % Positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>94.08</td>
</tr>
<tr>
<td>CD133</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3.5: Demonstrating sample flow cytometric data from which the percentage of CD44-positive cells present in the DU145 cell line was determined. Flow cytometry was performed using a FITC-conjugated isotype control to gate the cells and a FITC-conjugated anti-CD44 antibody to identify the population of cells expressing CD44. A histogram overlay of isotype versus test is also shown. The positive population was defined as cells present in gate B (CD44-FITC) and was calculated by subtracting the percentage of cells in gate A (iso-FITC) from the percentage in gate B (CD44-FITC). A total of 10,000 events were collected and analysed per sample. (n=3). Representative data.

Figure 3.6: Demonstrating sample flow cytometric data from which the percentage of CD133-positive cells present in the DU145 cell line was determined. Flow cytometry was performed using an APC-conjugated isotype control to gate the cells and an APC-conjugated anti-CD133 antibody to identify the population of cells expressing CD133. A histogram overlay of isotype versus test is also shown. The positive population was defined as cells present in gate C (CD133-APC) and was calculated by subtracting the percentage of cells in gate D (iso-APC) from the percentage in gate C (CD133-APC). A total of 10,000 events were collected and analysed per sample. (n=2). Representative data.
Therefore, using this technique, neither PC3 nor DU145 cells were found to contain a population of CD133-expressing cells. As such, other techniques were explored in attempt to identify putative CSCs expressing the CD133 antigen.

### 3.2.3. Profiling of Prostate Cancer Cell Lines by Immunofluorescence

The origin of prostate cancer stem cells, with regards to basal or luminal precursors, is somewhat controversial with several studies providing conflicting data (Maitland et al., 2011). Overall, most evidence suggests that human prostate cancer stem cells reside in the basal compartment and are of basal origin (Oldridge et al., 2012).

During this study the prostate cancer cell lines; PC3, DU145 and P4E6 were examined by immunofluorescence for the expression of markers associated with prostate stem cells, intermediate prostate cells, basal prostate cells and secretory luminal prostate cells.

Table 3.3 identifies the profiles of some of the cell types found in the human prostate according to Richardson et al., 2003; Collins et al., 2005 and Kasper, 2008.

*Table 3.3 Demonstrating the prostate cell marker profiles and corresponding cell types they identify*

<table>
<thead>
<tr>
<th>Marker Profile</th>
<th>Corresponding Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>P63- CD133+</td>
<td>Prostate stem cell</td>
</tr>
<tr>
<td>CD44+ CD133+</td>
<td>Prostate stem cell</td>
</tr>
<tr>
<td>AR+ PSCA+*</td>
<td>Intermediate cell</td>
</tr>
<tr>
<td>CD44+CD24+</td>
<td>Intermediate cell</td>
</tr>
<tr>
<td>CD44+ CD24-</td>
<td>Basal cell</td>
</tr>
<tr>
<td>CD44- CD24+</td>
<td>Luminal cell</td>
</tr>
<tr>
<td>AR+ CD24+</td>
<td>Luminal cell</td>
</tr>
</tbody>
</table>

*PSCA – prostate stem cell antigen, AR – androgen receptor*
Figures 3.7 to 3.9 demonstrate that the only marker to be consistently expressed across the three prostate cancer cell lines was CD44. In keeping with the flow cytometric data, the majority of PC3 and DU145 cells observed by immunofluorescence expressed high levels of this protein. This was also apparent with P4E6 cells. In addition to CD44, DU145 cells expressed high levels of CD24 (Figure 3.8). Therefore, the dual CD44/CD24 positivity of this cell line indicated that it demonstrated an intermediate phenotype. Using this antibody panel PC3 and P4E6 cells only expressed CD44, therefore, the majority of cells in these cell lines fitted the profile of a basal cell. As most evidence suggests that CD133-positive prostate cancer stem cells reside in the basal compartment, these cell lines may have contained a population of cancer stem cells. However, a lack of CD133 staining across all cells examined gave evidence to the contrary.

It is important to acknowledge that, despite efforts to identify an in-house cell line with a distinct population of CD133-expressing cells (data not shown), a suitable positive control was not available to test the validity of the antibody used in this study. The P4E6 cell line, kindly donated by Professor Norman Maitland from The University of York, had been previously shown to contain a very small population of CD133-positive cells, however, this cell line did not prove to be a suitable positive control as it did not contain a large enough population of CD133-expressing cells.

Isotype control staining revealed that a minimal level of background staining was observed for all three cell lines (See Appendix I). Hence, the staining patterns observed for the CD44 and CD24 antibodies were considered genuine.
Figure 3.7: Dual immunofluorescent staining of PC3 cells with several markers to identify cell types. Secondary antibody labelling was performed using AlexaFluor® antibodies. Nuclear staining was achieved using mounting media with DAPI. (n=3). Representative images. Image magnification at x20.
Figure 3.8: Dual immunofluorescent staining of DU145 cells with several markers to identify cell types. Secondary antibody and nuclear staining were achieved as described in Figure 3.7. (n=3). Representative images. Image magnification at x20.
Figure 3.9: Dual immunofluorescent staining of P4E6 cells with several markers to identify cell types. Secondary antibody and nuclear staining were achieved as described in Figure 3.7. (n=3). Representative images. Image magnification at x20.
According to the panel of markers defined in Table 3.3, this technique failed to identify a population of putative cancer stem cells in any of the cell lines examined. Table 3.4 summarises the results from the screening of these cell lines.

Table 3.4 Summarising the expression of the various antigens by PC3, DU145 and P4E6 cells and the proposed cell types based on their marker profiles by immunofluorescence.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>DU145</th>
<th>PC3</th>
<th>P4E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>P63</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD133</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD44</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AR</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PSCA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD24</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HLA-G</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proposed cell type</td>
<td>Intermediate</td>
<td>Basal</td>
<td>Basal</td>
</tr>
<tr>
<td>Putative CSC’s observed?</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

These results were consistent with those obtained by flow cytometric analysis of PC3 and DU145 in that most of the cells examined expressed CD44 however CD133 expression was not detected on any of the cells.
3.2.4. Attempted Isolation of CD133-positive P4E6 cells by MACS

Despite an inability to identify CD133-positive cells by flow cytometry and IF, magnetic activated cell sorting (MACS) was attempted as a means to isolate putative prostate cancer stem cells. It was believed that having enriched for this population, it would be easier to identify putative PCSCs by flow cytometry and IF. This approach was adopted because CD133-expressing cells have been reported to represent as few as 0.01% of cells in a cell line hence, could have been below the threshold for detection without prior enrichment (Pfeiffer and Schalken, 2010).

To that end, approximately 1x10⁸ P4E6 cells were subjected to MACS of CD133-positive cells. This cell line was chosen as our collaborators had previously identified and isolated CD133-positive putative CSCs using the same techniques. This technique involved labelling P4E6 cells with CD133 antibodies, conjugated with paramagnetic beads, prior to subjecting them to a magnetic field by running them through a magnetically-charged column. Antibody-bound cells expressing CD133 should have remained in the column while negative cells were able to pass through. The positive fraction was then purged from the column and subsequently analysed along with the negative and mixed fractions.

Unfortunately, despite several attempts (data not shown), the success of this technique could not be validated by flow cytometry due to the very low yield of putative CD133-positive cells. Furthermore, despite the use of different antibodies, immunofluorescence repeatedly failed to identify CD133-positive cells in any of the fractions (Figure 3.10 C). In addition, culturing the “positive fraction” of cells prior to examination (in order to allow them to recover from the isolation procedure) did not assist with the identification of CD133-positive cells in this cell line.
Figure 3.10: Dual immunofluorescent staining of non-selected P4E6 cells (A), CD133-negative P4E6 cells (B), and putative CD133-positive cells (C). Cells were subjected to MACS of CD133-positive cells and stained immediately by immunofluorescence. Secondary antibody and nuclear staining were achieved as described in Figure 3.7. (n=3). All stained cells were visualised. Representative images. Image magnification at x20.
3.2.5. Attempted Isolation of CD133-Positive Primary Prostate Cancer Cells by MACS

The CD133 molecule has been shown to be extremely sensitive to cell culture conditions (Maitland et al., 2011). As such, the failure to isolate CD133-positive cells from the P4E6 cell line may have been due to variation in the expression of CD133, as a result of differences in the media composition (such as foetal calf serum) between our lab and the lab at The University of York.

To establish whether this method of isolating putative prostate cancer stem cells was more appropriate for cultured primary cells, primary cultures of epithelial cells derived from patients with prostate cancer were established for the first time in our labs (Figure 3.11) (please refer to Chapter 7 for further studies with the primary cells). For the purpose of isolating putative prostate CSCs, these cells were primarily selected based on their rapid adhesion to collagen, to select for integrin α2β1hi cells, and were maintained with specialised media and irradiated murine fibroblast feeder cells (See Section 2.2.1.2). These conditions were optimised by the York group to promote self-renewal and inhibit differentiation of prostate cancer stem cells. Furthermore, the components of the defined media had been shown by this group not to interfere with the expression of CD133.

As with the P4E6 cells, the cultured prostate cancer-derived epithelial cells were subjected to MACS of CD133-positive cells and were subsequently examined by immunofluorescence. However, due to the slow growth of primary prostate epithelial cultures, only approximately 2x10⁷ cells were subjected to MACS hence, the yield was extremely low (fewer than 1000 cells were obtained for each repeat of the experiment). Unfortunately, despite multiple attempts and concurrent with what was observed with P4E6 cells, these methods failed to isolate and identify a population of cells expressing the CD133 molecule (Figure 3.12).
Figure 3.11: Illustrating the work flow for the attempted isolation of CD133-positive, putative prostate cancer stem cells (CSCs). Patient tissue was mechanically dissociated and digested overnight in collagenase solution prior to separating the stromal fraction from the epithelial fraction in a series of centrifugation steps. Epithelial cells were subjected to further selection based on rapid adhesion to collagen I-coated dishes. Those which bound to the collagen-coated dishes within 20 minutes belonged to the integrin α2β1 high fraction and were cultured with irradiated murine fibroblast feeder cells until they reached confluency, after which time they were subjected to magnetic-activated cell sorting (MACS) of CD133-positive cells. The red cross symbolises the failure to confirm the isolation of CD133-positive, putative prostate CSCs by immunofluorescence.
Figure 3.12: Dual immunofluorescent staining of non-selected primary prostate cancer epithelial cells (A), CD133-negative primary prostate cancer epithelial cells (B), and putative CD133-positive prostate cancer epithelial cells (C). Cells were subjected to MACS of CD133-positive cells and stained immediately by immunofluorescence. Secondary antibody and nuclear staining were achieved as described in Figure 3.7. (n=3). All stained cells were visualised. Representative images. Image magnification at x20.
3.2.6 Summary

Using the methods presented, this study failed to identify and isolate putative prostate cancer stem cells, with the marker profile reported by Collins et al., 2005, from three cell lines (PC3, DU145 and P4E6) and primary cultures of prostate cancer epithelia.

3.3 Discussion

Recent evidence supports the hypothesis that prostate cancer stem cells are responsible for prostate cancer initiation and progression (Collins et al., 2005; Collins and Maitland, 2006; Lawson and Witte, 2007; Signoretti and Loda, 2007; Kasper, 2008a; Kasper, 2008b, Goldstein et al., 2010; Tu and Lin, 2012). This study attempted to exploit the prostate cancer stem cell markers, previously established by Collins et al., 2005, to identify and isolate prostate cancer stem cells from androgen independent prostate cancer cell lines and primary epithelial cultures, derived from prostate cancer tissue. This was in an effort to further characterise these cells with a view to investigating their immunological status and expression of cancer-associated antigens to ascertain if prostate cancer stem cells (PCSCs) represent viable targets for immunotherapy. However, using the methods described, this study failed to identify and isolate putative cancer stem cells from cell lines and primary cultures.

Quantitative real-time PCR revealed that PC3 and DU145 cells expressed very low levels of CD133 at the mRNA level. This finding was as could be anticipated for a mixed population of prostate cancer cells which had not been enriched for putative cancer stem cells, since CD133 has been reported to be expressed by only a minute population of prostate cancer cells in cells lines and primary cultures (Collins et al., 2005; Wei et al., 2007). This technique also revealed that PC3 and DU145 cells expressed CD44 at the mRNA level, a molecule largely implicated in cancer progression and metastasis (Naor et al., 2008). Hence, quantitative real-time PCR demonstrated that PC3 and DU145 cells expressed two of the reported prostate cancer stem cell-associated markers at the mRNA level. However, since expression at the mRNA level does not confirm that the protein is functionally expressed, further investigation into the expression of these antigens was conducted at the protein level.
Flow cytometry is widely used in the field of cancer stem cell research as it enables identification of minute populations from large, mixed populations, using defined markers. During this study, the cell-surface expression of CD44 and CD133 by PC3 and DU145 cells was investigated by flow cytometry. This technique revealed that the majority of PC3 and DU145 cells expressed the CD44 antigen. As CD44 has been largely implicated in metastasis, this finding was in keeping with the fact that these cell lines were both derived from metastatic lesions (Naor et al., 2008; Orian-Rousseau, 2010). In addition, CD44 has been reported to identify transit-amplifying prostate cells and prostate cells of basal origin, therefore, this finding revealed that these cell lines possessed a basal/transit-amplifying signature as opposed to a luminal phenotype. The expression of basal markers in primary cultures and cell lines derived from prostate cancer is a common yet paradoxical finding since prostate cancers are known to lack cells with a basal phenotype (the absence of the basal-specific marker P63 is used by histologists to score prostate cancers) and mostly express luminal markers such as androgen receptor (AR) and prostate specific antigen (PSA) (Miki and Rhim, 2008).

During this study, CD133-positive cells were not detected by flow cytometry in the PC3 and DU145 cell lines. This finding however, did not confirm the absence of a cancer stem cell population in these cell lines as this approach suffers from the fact that during processing, procedures such as trypsinization may cleave many of the surface markers thus, rendering them unavailable for antibody binding (Singh et al., 2012). Moreover, CD133 is arguably an unreliable marker for prostate cancer stem cells since its expression may not be restricted to cancer stem cells and even CD133-negative cells have been shown to be tumourigenic (Shmelkov et al., 2008).

Recently, a large study involving six prostate cancer cell lines, DU145, 22Rv1, LAPC-4, DuCaP, LNCaP, and PC-3, failed to identify a population of CD133-positive cells in all but DU145 cells (0.01%) by flow cytometry (Pfeiffer and Schalken, 2010). Therefore, the inability to identify a population of CD133-positive cells in the PC3 cell line was consistent with the findings of this group. Furthermore, their study also discredited CD133 as a maker of CSCs in cell lines, based on the finding that the five CD133-negative cell lines demonstrated other properties of cancer stem cells (Pfeiffer and Schalken, 2010). In addition to flow cytometry, which identified a population of CD133-positive cells in the DU145 cell line, Pfeiffer and Schalken (2010) also examined DU145 cells by
immunohistochemistry. Interestingly, using this technique they failed to identify CD133-
expressing cells therefore, inconsistencies in the expression of the CD133 molecule were
apparent within the same study.

In an attempt to characterise the prostate cancer cell lines PC3, DU145 and P4E6 and
determine whether they contained a population of putative PCSCs, this study also
examined the expression of CD44, CD133 and several other markers by
immunofluorescence. Using this technique, the only marker found to be consistently
expressed among the three cell lines was CD44. This finding was in keeping with the
results obtained for PC3 and DU145 using flow cytometry. Although these three cell lines
contained large populations of cells expressing the basal marker CD44, according to this
study, they did not contain cells expressing the basal marker P63. It is however, important
to acknowledge that the P63 antibody used in this study was withdrawn from Abcam after
this work was conducted, which may have been due to its inefficacy. As such, this result
was possibly unreliable.

Immunofluorescence failed to identify the luminal markers AR, PSCA and CD24 in PC3
and P4E6 cells, thereby confirming that these cell lines comprised cells with a basal
phenotype. The majority of DU145 cells however, appeared to be dual-positive for the
expression of CD44 and CD24 thus, demonstrating a transit-amplifying/intermediate
phenotype.

As previously described (See Section 1.1.3.5), HLA-G is an immunomodulatory molecule
that has been shown to be expressed by stem cells and putative cancer stem cells, among
other cell types (Brown et al., 2012; Morandi et al., 2012). It was important to investigate
the expression of this molecule as the presence of HLA-G on putative prostate cancer stem
cells would have serious implications when considering these cells as targets for
immunotherapy. Therefore, in an attempt to ascertain whether putative (CD133-positive)
prostate cancer stem cells express this immunomodulatory molecule, PC3, DU145 and
P4E6 cells were dual-stained with HLA-G and CD133 antibodies. In this study, none of
these cell lines were found to contain a population of cells expressing HLA-G. Furthermore,
this technique also failed to identify cells expressing the CD133 molecule. Hence, as with flow cytometry, putative prostate cancer stem cells were not identified in
any of the three prostate cancer cell lines examined using immunofluorescence.
As CD133-positive putative CSCs have been reported to comprise a very small population of cells (Collins et al., 2005; Wei et al., 2008), enrichment of these cells, prior to conducting flow cytometric/immunofluorescence analysis, was attempted using magnetic activated cell sorting (MACS). However, this technique proved to be problematic for a number of reasons namely; too few cells were isolated to conduct flow cytometry, many cells died during the procedure and the majority of the isolated cells had to be sacrificed for immunofluorescence to confirm/disprove the success of the isolation procedure. Furthermore, this technique appeared to be unsuccessful as CD133-positive cells could not be identified from a cell line with a known, albeit small, population of CD133-expressing cells (P4E6), or primary cultures of epithelial cells derived from prostate cancer tissues.

It is important to acknowledge that differences in culture conditions and the methods of harvesting and fixing cells may affect the expression of molecules such as CD133, hence, may account for the inconsistencies observed between the other groups and this study. Recently, using glioblastoma stem cells as a model, Sherman and colleagues (2011) revealed that the CD133 molecule is highly sensitive to fixation methods, which may provide an explanation as to why this molecule was not identified in any of the three cell lines or primary cell cultures during this study. Throughout this study, cells were routinely fixed with 4% paraformaldehyde prior to conducting immunofluorescence. Hence, this procedure may have damaged the CD133 molecule, thereby affecting antibody binding and resulting in false-negative results. Furthermore, as too few cells were obtained for flow cytometric analysis following magnetic activated cell sorting (MACS), immunofluorescence was routinely exploited in attempt to confirm the success of the isolation procedure. However, fixation of the cells following MACS may have led to false-negative results, implying that the procedure had failed or that the cell lines did not contain a population of CD133-positive cells. Unfortunately, Sherman et al., (2011) released their paper more than two years after the experiments were conducted in this study, as such, time did not allow this issue to be addressed. Furthermore, as a positive control for the expression of CD133 had not been identified for the purpose of this study (data not shown) it was not possible to verify that the antibodies and techniques used were suitable for the detection of this molecule. Moreover, in addition to the CD133 molecule being extremely sensitive to culture, harvesting and fixing conditions, the availability of CD133 antibodies was largely limited to those which identified only glycosylated forms of CD133 (Osmond et al., 2010).
These facts emphasise the problems with exploiting CD133 as a marker to identify putative prostate cancer stem cells. In addition to the aforementioned issues, long term \textit{in vitro} passaging may also result in downregulation of the CD133 molecule, hence account for differences observed between cell lines, primary cells and tissue sections. Therefore, the expression of CD133 is dynamic and easily influenced by environmental changes. As such, when expressed, this molecule may well identify putative prostate cancer stem cells however, an absence of this molecule does not necessarily imply an absence of a putative PCSC population. Consequently, the usefulness of this molecule as a marker of putative PCSCs is greatly limited by its sensitivity hence, there is a great need to identify more robust markers which may be exploited for the identification and isolation of putative prostate cancer stem cells.

In light of the problems encountered during the study of putative CD133-expressing PCSCs, it was necessary to adopt an alternative approach to investigating cancer stem cells in prostate cancer. In 2008, Mani and colleagues merged the field of cancer stem cells and epithelial to mesenchymal transition when they demonstrated that EMT generated cells with the properties of cancer stem cells in human breast cancer (Mani \textit{et al}., 2008). Since their discovery, this topic raised a great deal of attention and consequently, several others have provided further evidence for a link between EMT and CSCs in other cancers (Fazul \textit{et al}., 2009; Santiseban \textit{et al}., 2009; Kong \textit{et al}., 2010). Since cancer cells transitioning via EMT are easily identified using a panel of several markers, the investigation of this phenomenon and the acquisition stem-like attributes proved to be another approach for investigating the properties of prostate cancer stem cells. Hence, further studies undertaken throughout the duration of this project sought to investigate the properties of EMT and ascertain whether or not this phenomenon generates cells with the properties of stem cells in human prostate cancer.
Chapter 4:

Derivation of a Model for the Investigation of Epithelial to Mesenchymal Transition in Human Prostate Cancer

4.1 Introduction

In recent years, epithelial to mesenchymal transition has emerged as a key concept in cancer biology as it has been evidenced to play a critical role in cancer invasion and metastasis. Although the cancer stem cell hypothesis and work in this field to date may provide evidence to address the cell of origin of a given tumour and provide explanations for tumour relapse following therapy, the causative role of migrating cancer stem cells in metastasis remains to be proven (Sampieri and Fodde, 2012). However, cancer stem cells are often implicated in metastasis due to the consensus that they are unique in their ability to propagate tumours due to their inherent self-renewal capabilities. Hence, the ability to form secondary metastases is believed by many to be restricted to cancer stem or cancer stem-like cells. The discovery that epithelial to mesenchymal transition generates cells with the properties of stem cells provided a greater understanding of the processes underlying tumourigenesis. Acquisition of mesenchymal traits by carcinoma cells via EMT facilitates invasion, via increased production of matrix-degrading MMPs, and metastasis, via decreased expression of adhesion molecules with a concomitant increase in the expression of molecules conferring motility, and the acquisition of stem cell traits confers self-renewal capabilities necessary for the formation of macrometastases (Mani et al., 2008; Bertram, 2012). Previous work, conducted during this project, failed to identify CD133-positive putative PCSCs therefore, it was necessary to approach the topic of prostate CSCs from another angle. Although the phenomenon of EMT and the generation of cancer stem-like cells has been largely studied in the context of breast cancer, further work in this area is required in the field of prostate cancer.
4.2 Results

4.2.1 Identification and Selection of a Model Cell Line for the Investigation of Epithelial to Mesenchymal Transition

In order to investigate the phenomenon of epithelial to mesenchymal transition in human prostate cancer, it was necessary to identify and select a model cell line, demonstrating evidence of this phenomenon, on which to conduct further studies. To that end, five androgen-independent human prostate cancer cell lines, two from metastatic lesions and three derived from primary tissues, were selected and screened using immunofluorescence on a panel of several EMT and CSC-associated markers. Phenotypic differences in cellular morphology between the cell lines were clearly observable microscopically (Figure 4.1), therefore, it was speculated that they would exhibit unique marker expression profiles.

DU145 cells demonstrated a tight cobblestone morphology, characteristic of epithelial cells, whereas PC3 and OPCT-2 cells appeared to demonstrate mesenchymal-like phenotypes with loose cell-cell contact and spindle-like (fibroblastoid) morphologies. P4E6 cells appeared similar to DU145 cells. The OPCT-1 cell line appeared to comprise a mixed population of both epithelial cells, forming colonies with tight cell-cell adhesion and mesenchymal cells, with spindle-like morphology present between the colonies.

Figure 4.1: Bright field images of human prostate cancer cell lines derived from metastatic lesions; DU145 and PC3, and primary tissues; P4E6, OPCT-1 and OPCT-2. Image magnification at x10.
Figures 4.2 to 4.11 demonstrate the results of the IF screening which identified the OPCT-1 cell line as a model for the investigation of EMT in human prostate cancer. In parallel to staining with test antibodies, appropriate isotype controls were used to gauge the level of background staining and confirm that any staining observed was as a result of specific antibody-antigen binding activity (See Appendix IV).

Vimentin is a ubiquitous intermediate filament protein expressed by all primitive cells, however, upon differentiation most non-mesenchymal cell types replace vimentin with other intermediate filaments, such as cytokeratins. As such, vimentin is perhaps the most common marker used to identify cells of mesenchymal origin (Korita et al., 2010). Therefore, throughout this study antibodies directed against vimentin were widely used to identify putative mesenchymal cells, which may have arisen as a result of EMT. Figures 4.2 to 4.5 confirm that vimentin was detected in all of the five cell lines, however the expression patterns varied markedly, hence, clear differences in the staining intensity, distribution and frequency of vimentin expression were observed among the cell lines. The metastasis-derived cell lines, PC3 and DU145, and the primary cell line P4E6 demonstrated focal vimentin expression in a high proportion of the cells. Conversely, the primary cell lines, OPCT-1 and OPCT-2, demonstrated differential vimentin expression, with a sophisticated network of vimentin fibres throughout some cells, lower levels of vimentin appearing around the nucleus in other cells and a majority of cells which did not stain positive for this intermediate filament.

E-Cadherin, one of several cell adhesion molecules (CAMs), is a key component in the formation of cell-cell adherens-type junctions in epithelial tissues. As such, this protein is widely used to identify cells of epithelial origin. In normal epithelia one would observe E-Cadherin expression at the cell surface, between cells (van Roy and Berx, 2008). However, E-Cadherin mediated cell-cell adhesion is lost during the development of most epithelial cancers and changes in E-Cadherin expression (from the membrane to the cytoplasm) have been strongly associated with aggressive, metastatic cancer (Christofori and Semb, 1999; Guzińska-Ustymowicz et al., 2004). During this study, antibodies directed against E-Cadherin were used as a means to identify epithelial cells and distinguish them from mesenchymal cells within the prostate cancer cell lines. However, consistent with previous observations of epithelial cancers, E-Cadherin was atypically expressed in all five cell lines as, despite being expressed by the majority of cells, an
accumulation of this protein at the cell surface, between the cells was not evident in any of the cell lines examined (Figure 4.2).

Cytokeratins are a family of intermediate filament proteins which are primarily expressed by differentiated epithelial cells. In fact, they are generally considered to be the most fundamental markers of epithelial differentiation and the composition of cytokeratins is known to reflect both the cell type and differentiation status (Moll et al., 1982). In the prostate, basal and luminal cells demonstrate differential expression of cytokeratins and these can be exploited to identify these cells from a mixed population. As cytokeratins are produced in cell lineage dependent patterns and these patterns are reproduced with great fidelity, even in carcinomas, they are widely used in pathologic diagnosis and represent robust markers for cells of epithelial origin (Nagle, 1994). For the purpose of this study, pan cytokeratin antibodies were exploited, in addition to E-Cadherin antibodies, as a means to identify and distinguish epithelial cells from mesenchymal cells in the prostate cancer cell lines. As expected, cytokeratins were expressed by the majority of the cells in each of the cell lines tested (Figure 4.3).

The use of epithelial markers, such as E-Cadherin and cytokeratins, in conjunction with vimentin enabled the identification of epithelial, mesenchymal and mixed populations within the cell lines. Indeed, the co-expression of epithelial and mesenchymal markers such as vimentin and cytokeratins is not uncommon and has been observed previously in both benign and malignant prostatic epithelia (Leong et al., 1988). Despite marked differences in cell morphology, Figures 4.2 and 4.3 demonstrate that the metastasis-derived cell lines, DU145 and PC3, exhibited similar, mixed phenotypes with a high proportion of cells expressing both epithelial and mesenchymal markers. The same was only true of one of the primary-derived cell lines, P4E6, although the OPCT-2 cell line also contained a small population of dual-positive cells. OPCT-1 was the only cell line to demonstrate distinct populations with regards to E-Cadherin/cytokeratins and vimentin expression (evidenced by single-positive staining which is indicated by the orange arrows in Figures 4.2 and 4.3). However, this cell line also contained a population of dual-positive cells. That is to say, the OPCT-1 cell line contained cells with properties of epithelial cells (single positive for E-Cadherin or cytokeratins), cells with properties of mesenchymal cells (single positive for vimentin) and cells with a mixed phenotype (dual positive for epithelial and mesenchymal markers). Furthermore, the single vimentin-positive cells in this cell line grew in isolation and demonstrated fibroblastoid morphologies, consistent with cells of
mesenchymal origin, whereas the single E-Cadherin/cytokeratin-positive cells formed colonies of cells, consistent with cells of epithelial origin. The dual-positive cells in this cell line may have represented cells which were transitioning between states.

Beta-Catenin is a multifunctional protein which can be located in three cell compartments, namely the plasma membrane, where it associated with E-Cadherin, the cytoplasm and the nucleus (Orsulic et al., 1999). Nuclear accumulation of β-Catenin has been associated with induction of EMT due to its downstream effects on several EMT-inducing transcription factors (Vincan and Barker, 2008; Polyak and Weinberg, 2009). Therefore, the location of β-Catenin within a cell may reflect the cell status with regards to EMT. Figure 4.4 demonstrates that cytoplasmic β-Catenin staining was observed in all five cell lines however, membranous staining was only clearly observed in DU145 cells, as indicated by the white arrows. The green arrows indicate nuclear staining which was most evident in the DU145 and OPCT-1 cells which also expressed vimentin. The concomitant expression of nuclear β-Catenin and vimentin was consistent with the fact that accumulation of β-Catenin in the nucleus is known to induce EMT. However, it is important to acknowledge that the subcellular localisation of staining is not accurate when imaging samples with a fluorescence microscope and confocal microscopy would be required for this purpose.

Cellular fibronectin is an extracellular matrix protein which is known to play a key role in cell proliferation, adhesion and migration. This protein is primarily produced and secreted by mesenchymal cells and fibroblasts hence, has been widely utilised as a mesenchymal marker and in studies of EMT (Mani et al., 2008; Phadnis et al., 2009; Araida et al., 2010). As this protein has been shown to be produced by other cell types, including epithelial cells, it was important to investigate the expression of cellular fibronectin in conjunction with vimentin (Kolachala et al., 2007). The expression of fibronectin by vimentin-positive cells in a prostate cancer cell line provides compelling evidence of epithelial to mesenchymal transition as both proteins are characteristic of mesenchymal cells. Furthermore, the expression of said proteins not only implies a phenotypic change in the transdifferentiated cells but also a functional modification. Of the five prostate cancer cell lines examined, only OPCT-1 and OPCT-2 contained a population of cells expressing fibronectin (Figure 4.5). The majority of cells which expressed fibronectin in the OPCT-1 cell line co-expressed vimentin whereas, fibronectin expression was not confined to vimentin-positive cells in the OPCT-2 cell line.
Figure 4.2: Dual immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a murine monoclonal antibody against the epithelial marker, E-Cadherin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. E-Cadherin was labelled with an anti-mouse Alexa Fluor®568-conjugated secondary antibody and vimentin was labelled with an anti-rabbit Alexa Fluor®488-conjugated secondary antibody. Nuclear staining was achieved using mounting media with DAPI. (n=3). Representative images. Image magnification at x20. Arrows demonstrate the epithelial and mesenchymal cell types present in the OPCT-1 cell line.
**Figure 4.3:** Dual immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a murine pan polyclonal antibody against the epithelial marker, cytokeratin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=3). Representative images. Image magnification at x20. Arrows demonstrate the epithelial and mesenchymal cell types present in the OPCT-1 cell line.
**Figure 4.4:** Dual immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a murine monoclonal antibody against Beta-Catenin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2). Representative images. Image magnification at x20. The green arrows indicate nuclear Beta-Catenin staining. The white arrow indicates membranous expression of Beta-Catenin.
Figure 4.5: Dual immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a murine monoclonal antibody against cellular fibronectin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2). Representative images. Image magnification at x20.
N-Cadherin, another member in the family of CAMs, is typically expressed by mesenchymal lineage cells (such as adipocytes, chondrocytes, osteoblasts and myoblasts), fibroblasts, endothelial cells and neurons (Wever and Mareel, 2003; Zeisberg and Neilson, 2009). Contrary to E-Cadherin, N-Cadherin has been shown to be upregulated in several carcinomas and has been implicated in invasion, migration and progression to castration resistance in prostate cancer (Derycke and Bracke, 2004; Tanaka et al., 2010). As this molecule is expressed by a variety of healthy cell types, its expression on carcinoma cells is believed to facilitate perineural and muscular invasion as well as transendothelial migration by mediating adhesion to neural, muscular and endothelial cells (Maret et al., 2010). Furthermore, this molecule has been associated with epithelial to mesenchymal transition and the so-called “cadherin switch”, from E–Cadherin to N-Cadherin, is often used to monitor the EMT process in both embryonic development and cancer progression (Zeisberg and Neilson, 2009). In light of this, the expression of N-Cadherin was examined in the five prostate cancer cell lines (Figure 4.6). Interestingly, the expression of this molecule varied markedly among the cells as only PC3, P4E6 and OPCT-1 cells demonstrated N-Cadherin expression. Of particular interest was the observation that this protein appeared to be expressed at a higher level on the cells with fibroblastoid morphology in the OPCT-1 cell line. Furthermore, the PC3 cell line, which exhibits fibroblastoid morphology, demonstrated the highest intensity of N-Cadherin staining overall.

FSP1 is a cytoplasmic filament-binding S100 protein which has been reported to be uniquely expressed in fibroblasts (Strutz et al., 1995; Okada et al., 1998). Furthermore, FSP1 has been shown to be expressed during epithelial to mesenchymal transitions in vivo and it has been employed to identify cells which have fully transitioned from epithelial cells to fibroblasts (Okada et al., 1997). During this study, the five prostate cancer cell lines were screened for this marker in attempt to ascertain whether the vimentin/fibronectin-positive cells, identified in previous immunochemical tests, were fibroblasts which had arisen as a result of EMT. However, Figure 4.7 demonstrates that FSP1 was not detected in any of the prostate cancer cell lines tested. Therefore, the identity of the vimentin-positive cells remained elusive.

In order to confirm that the absence of staining was not due to inefficacy of the antibody, the FSP1 antibody was tested on the murine fibroblast cell line, NIH 3T3 (Figure 4.12).
Figure 4.6: Immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a murine monoclonal antibody against N-Cadherin. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2) Representative images. Image magnification at x20.
Figure 4.7: Immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a rabbit polyclonal antibody against fibroblast-specific protein 1 (FSP1). Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2). Representative images. Image magnification at x20.
During this study, the five prostate cancer cell lines were examined for the expression of three zinc-finger transcription factors which have been widely reported to play pivotal roles in the induction of epithelial to mesenchymal transition.

Snail and Slug (Snai2) are two of several transcription factors known to directly repress the expression of E-Cadherin hence, are potent inducers of EMT (Savagner et al., 1997; Haraguchi et al., 2009; Casas et al., 2011). Ectopic expression of either of these transcription factors has been shown to be sufficient for EMT induction hence, several studies have artificially induced EMT in this manner (Mani et al., 2008; Bolos et al., 2003). In addition to inducing EMT, these transcription factors have been shown to repress the transcription of several factors involved in apoptosis and as such, have been implicated in the mediation of chemo- and radio-resistance observed in cells which have undergone EMT (Haraguchi, 2009; Kurrey et al., 2009). During this study, the expression of Snail and Slug was investigated in the five prostate cancer cell lines in an attempt to identify cells undergoing EMT. Figures 4.8 and 4.9 illustrate that only the DU145 and OPCT-1 cell lines contained populations of cells expressing Snail and Slug. Please note, as the Slug staining of PC3 cells appeared ambiguous it was likely to have been non-specific. In contrast to OPCT-1, which contained very small populations of cells expressing Snail or Slug, a large proportion of DU145 cells appeared to express these transcription factors. Interestingly, rather than being confined to the nucleus, positive staining was observed in the cytoplasm of DU145 and OPCT-1 cells. This observation was consistent with other studies which have also reported the expression of Snail in the cytoplasm (Dominguez et al., 2003; Luo et al., 2012). Furthermore, the “dotted staining” pattern reported by Dominguez et al., (2003) was also observed in these studies.

Twist, another of the zinc-finger transcription factors known to repress E-Cadherin transcription and induce EMT, has been shown to play an essential role in tumour metastasis. In contrast to Snail and Slug, Twist is an indirect repressor of E-Cadherin and has been shown to directly induce the expression of Slug in order to initiate EMT (Yang et al., 2004). As with Snail and Slug, ectopic expression of Twist has been shown to induce EMT in vitro (Mani et al., 2008). The expression of this key transcription factor was also investigated in the five prostate cancer cell lines however, Twist staining was not observed in any of the cells tested (Figure 4.10).
Figure 4.8: Immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a goat polyclonal antibody against the transcription factor Snail. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2) Representative images. Image magnification at x20.
Figure 4.9: Immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a goat polyclonal antibody against the transcription factor Slug. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2). Representative images. Image magnification at x20.
Figure 4.10: Immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a rabbit polyclonal antibody against the transcription factor Twist. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2). Representative images. Image magnification at x20.
In 2008, Mani et al., demonstrated that EMT of immortalised or transformed mammary epithelial cells generated cells with properties of stem cells and that the cells which had undergone this transition expressed high levels of CD44. As CD44 is one of the markers of putative prostate cancer stem cells and a connection between EMT and the generation of CSCs has been shown in breast cancer cells, the expression of this molecule was also investigated in the five prostate cancer cell lines. Figure 4.11 demonstrates that a high proportion of the cells in each of the prostate cancer cell lines examined expressed CD44. Two of the primary-derived cell lines, P4E6 and OPCT-2, appeared to exhibit the lowest staining intensities whereas the metastasis-derived cell lines, PC3 and DU145, and the primary-derived OPCT-1 cell line demonstrated the highest staining intensities.

Figure 4.12 illustrates the immunofluorescent staining of the murine fibroblast cell line, NIH 3T3, which served as a positive control for the FSP1, Snail and Slug antibodies used throughout this study. Although the efficacy of the FSP1 and Snail antibodies was confirmed using this cell line, very weak staining was observed with the Snail antibody.
Figure 4.11: Immunofluorescent staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 using a murine monoclonal antibody against CD44. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=3). Representative images. Image magnification at x20.
Figure 4.12: Immunofluorescent staining of murine fibroblast NIH 3T3 cells as a positive control for the rabbit polyclonal antibody against FSP1 and goat polyclonal antibodies against Snail and Slug. Appropriate isotype controls are also shown. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2) Representative images. Image magnification at x20.
As anticipated, the five cell lines varied markedly with regards to the expression of several EMT-associated proteins. Furthermore, differential expression of proteins conferring properties of epithelial and mesenchymal cells was evident, particularly in the OPCT-1 cell line. The results from the immunofluorescent screening of the five prostate cancer cell lines are recapitulated in Table 4.1.

Table 4.1 Summarising the results of the IF screening of DU145, PC3, P4E6, OPCT-1 and OPCT-2 cells for the expression of several EMT-associated markers

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<th>Fibro</th>
<th>N-Cad</th>
<th>Snail</th>
<th>Slug</th>
<th>Twist</th>
<th>CD44</th>
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<td>-</td>
<td>-</td>
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</table>

+ denotes 1 or more positive cells were observed  
- denotes no positive cells were observed  
+ denotes that this cell line demonstrated expression of several EMT-associated markers

The OPCT-1 cell line was the only cell line investigated in this study which expressed all of the EMT-associated markers, with the exception of Twist (Table 4.1). Furthermore, this cell line was unique in its possession of morphologically distinct populations which could be observed by both light and fluorescence microscopy (Figure 4.13). Although several of the EMT-associated markers were detected in all five of the cell lines examined, only the OPCT-1 cell line demonstrated a clear distinction between epithelial and mesenchymal populations.

Therefore, immunofluorescent screening with a panel of EMT-associated antibodies identified the OPCT-1 cell line as model for further investigation of EMT in human prostate cancer. Figure 4.14 summarises the positive staining patterns of OPCT-1 cells labelled with some of the most common marker combinations used for the investigation of EMT and provides justification for conducting further studies on this cell line.
Figure 4.13: Bright field images and fluorescent micrographs of DU145, PC3, P4E6, OPCT-1 and OPCT-2 cells stained with E-Cadherin and vimentin. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. Representative images. Image magnification at x10 for BF images and x20 for fluorescent micrographs. The red rectangle identifies the OPCT-1 cell line as a suitable model for the investigation of EMT.
Figure 4.14: Summary composite of OPCT-1 stained with common markers used to investigate EMT; cytokeratin pan/vimentin, E-Cadherin/vimentin, fibronectin/vimentin. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. Representative images. Image magnification at x20.
4.2.2 Verification of the Observation of Epithelial to Mesenchymal Transition in OPCT-1 cells by Selective Cloning of the OPCT-1 Cell Line

Information regarding the derivation and characterisation of the OPCT cell lines is scarce and is limited to three abstracts from AACR Meetings held in 2004, 2005 and 2007 and the profiles provided on the Asterand website, from where they can be purchased (please refer to links in Appendix IV). Of concern was the notion that, rather than showing evidence of epithelial to mesenchymal transition, perhaps the OPCT-1 cell line was a mixed cell line comprised of immortalised stroma in addition to epithelial cells. Figure 4.15 demonstrates that a cell line with a mixed population of epithelial and mesenchymal (stromal) cells may have resulted if the epithelial cells had not been separated from the stromal cells prior to transformation with the HPV16/18 genes E6 and E7.

![Figure 4.15: Illustrating concerns that the OPCT-1 cell line may be comprised of immortalised epithelial and stromal cells rather than demonstrating evidence of epithelial to mesenchymal transition.](image)

In light of these concerns, it was necessary to establish whether the OPCT-1 cell line did contain a population of cells which were transitioning between epithelial and mesenchymal states. In order to achieve this, the OPCT-1 cell line was cloned using the limiting dilution assay whereby less than one cell was seeded per well into the wells of six 96-well plates. Previous attempts to isolate individual E-Cadherin-positive OPCT-1 cells, using live cell
laser capture microdissection failed however, cloning by limiting dilution yielded a total of 74 clones, which were microscopically examined to confirm they were derived from single cells. Figure 4.16 provides two examples of the individual colonies after 21 days of culture in the original 96 well plates in which they were cloned. This figure demonstrates that the OPCT-1 clones arose from single colonies of cells, which were derived from single cells.

![Figure 4.16: Demonstrating individual colonies of cloned OPCT-1 cells after 21 days in culture. Representative images of A) edge of colony B) centre of colony. x20 magnification.](image)

Of the 74 clones, 51 were expanded, frozen down in liquid nitrogen at three passages (passage 1, 2 and 3) and screened at the second passage (P2) for the expression of the epithelial marker E-Cadherin and the mesenchymal marker vimentin. In addition to examining these markers, the 51 clones were screened at the same passage for CD44 and CD24 to address a possible correlation with the CSC phenotype. Table 4.2 displays the results to the initial IF screening of the OPCT-1 clones.
Table 4.2 The expression of E-Cadherin, vimentin, CD44 and CD24 by 51 OPCT-1 clones screened by immunofluorescence

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<th>CD24</th>
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</tr>
<tr>
<td>P5A2</td>
<td>+++</td>
<td>++</td>
<td>Yes</td>
<td>+++</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>P5G11</td>
<td>+++</td>
<td>+</td>
<td>Yes</td>
<td>+++</td>
<td>-</td>
<td>n/a</td>
</tr>
</tbody>
</table>

+++ expressed by \(^1\) 10-100% of cells \(^\ast\) 1-10% of cells + expressed by \(^\ast\) <1% of cells – no positive cells observed
Yes = individual cells expressing both markers present No = only single-positive cells observed

Table 4.2 demonstrates that CD44 and E-Cadherin were both expressed by a high proportion of cells in each of the OPCT-1 clones. Large variation in the number of vimentin-positive cells within each clone was evident and vimentin was detected in all but two of the 51 clones. The expression of CD24 was minimal in the clones which expressed this protein.

Not only did these data confirm that populations of cells derived from single OPCT-1 cells gave rise to epithelial and mesenchymal cell types, the variation in the level of vimentin expression across the clones also revealed that individual OPCT-1 cells differed in their ability and frequency to transdifferentiate. Therefore, the OPCT-1 clones provided a range of phenotypes which could be exploited to investigate epithelial to mesenchymal transition in human prostate cancer. As such, 12 clones with differing phenotypes were selected for further interrogation. Please refer to Appendix IV for images of the 12 selected clones growing as single colonies in the 96-well plates in which they were cloned.

In order to assess the stability of the phenotypes of the 12 clones of interest, ascertained from the original screening of the 51 OPCT-1 clones (Table 4.2), further screening of E-Cadherin and vimentin expression was conducted by immunofluorescence. To that end, the clones were passaged, cryopreserved, re-cultured and re-screened on two separate occasions. Figures 4.17a and 4.17b display the results from these screens in addition to the results from the initial screening.
Figure 4.17a: Dual immunofluorescent staining of 1-6 of 12 OPCT-1 clones of interest; P5B3, P3E5, P2E9, P4C7, P5E4, P6D4 using a murine monoclonal antibody against the epithelial marker, E-Cadherin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. Secondary antibody and nuclear staining were achieved as per Figure 4.2. 1st screen represents the initial screening of the 51 clones, from which these clones were selected (P2). 2nd screen represents the second screening conducted after defrosting and passaging the cells (P3) and 3rd screen represents the third screening conducted after defrosting and passaging the clones for a second time (P4). (n=3). Representative images. Image magnification at x20. Yellow arrows indicate vimentin-positive cells and red arrows indicate that these two clones exhibited a change in phenotype.
Figure 4.17b: Dual immunofluorescent staining of 6 of 12 OPCT-1 clones of interest: P4F2, P4G10, P5F3, P3H8, P2B9 and P4B6. Stained as per the staining demonstrated in Figure 4.17a. 1st screen represents the initial screening of the 51 clones, from which these clones were selected (P2). 2nd screen represents the second screening conducted after defrosting and passaging the cells (P3) and 3rd screen represents the third screening conducted after defrosting and passaging the clones for a second time (P4). (n=3). Representative images. Image magnification at x20. Red arrow indicates that clone P4G10 exhibited a change in phenotype.
The majority of the 12 clones maintained the original phenotype, observed in the first screening (Figures 4.17a and 4.17b). However, during the third screening, vimentin-positive cells were detected for the first time in clones P5B3 and P3E5 (indicated by yellow arrows). Therefore, none of the clones were completely negative for the expression of vimentin. Clone P4G10 also showed an increase in vimentin expression during the third screening.

From the 12 clones of interest, five (presented in increasing order of vimentin expression) were selected for further studies; P5B3, P6D4, P5F3, P2B9, P4B6, in which they were interrogated and compared to parental OPCT-1 (Chapters 5 and 6). To ensure maintenance of phenotype, these clones were assayed only at passages 2-5 and were screened by immunofluorescence in parallel to each assay throughout the duration of further studies. Figure 4.20 demonstrates the morphology of the five selected clones and parental OPCT-1 observed using light microscopy.

![Figure 4.18: Bright field images of five OPCT-1 clones selected for further study and parental OPCT-1. Representative images. Image magnification at x10.](image)

Clear morphological differences between the clones are evident from Figure 4.18 where clone P5B3, the least vimentin-positive clone, exhibited cobblestone morphology and this morphology was gradually lost as the clones increased in vimentin positivity. Clone P4B6, the most vimentin positive clone, possessed a high proportion of fibroblastoid cells which surrounded colonies of epithelial-looking cells.
Figure 4.19 demonstrates the distinct vimentin and E-Cadherin expression profiles of the five OPCT-1 clones selected for further studies. These clones ranged from very low to high in terms of vimentin expression. It is worth noting that although a single vimentin-positive cell was detected in the 3rd screening of clone P5B3, vimentin-positive cells were extremely rare and often not detected during immunofluorescent screening of this clone.

These five clones provided a model which could be exploited to investigate the phenomenon of EMT in future in vitro and in vivo studies.
4.3 Discussion

Epithelial to mesenchymal transition is emerging as a vital process involved in the progression of early stage tumours towards malignancy. Furthermore, this process has been implicated in the generation of cancer stem-like cells which are believed to contribute significantly towards therapeutic resistance and tumour relapse (Mani et al., 2008; May et al., 2011). This phenomenon has been most extensively studied using breast cancer as a model, therefore, there is a need to investigate the role and properties of EMT in other human malignancies, including prostate cancer. The work documented in this chapter aimed to identify a suitable prostate cancer cell line from which clones could be derived for the investigation of epithelial to mesenchymal transition and the generation of cancer stem-like cells in human prostate cancer.

The five prostate cancer cell lines selected for the investigation of EMT; PC3, DU145, P4E6, OPCT-1 and OPCT-2, demonstrated distinct morphologies, when examined microscopically, which did not necessarily reflect their EMT status, as determined by immunofluorescence. However, despite morphological differences, a high proportion of the cells in each of the five cell lines expressed the putative cancer stem cell marker, CD44. Surprisingly, the metastasis-derived PC3 cell line, which exhibited a fibroblastoid, mesenchymal-like morphology, did not appear to express any of the investigated EMT-associated transcription factors, Snail, Slug and Twist, or fibronectin, an extracellular matrix protein produced and secreted by mesenchymal cells. Furthermore, PC3 cells expressed the epithelial markers E-Cadherin and cytokeratins, in addition to the mesenchymal markers N-Cadherin and vimentin. Therefore, this cell line exhibited an intermediate phenotype which was neither epithelial nor mesenchymal.

The metastasis-derived DU145 cell line however, which exhibited cobblestone morphology, typical of epithelial cells, demonstrated the highest staining intensities for Snail and Slug. As with the PC3 cell line, DU145 cells demonstrated an intermediate phenotype, characterised by the co-expression of the epithelial markers, E-Cadherin and cytokeratins and the mesenchymal marker vimentin. Unlike PC3 cells however, DU145 cells did not express the mesenchymal cell adhesion molecule (CAM), N-Cadherin. The DU145 cell line appeared to contain a population of vimentin-positive cells with nuclear
accumulation of β-Catenin, an EMT-associated phenotype, however, this must be verified using confocal microscopy. In keeping with the PC3 cell line, DU145 cells did not appear to express fibronectin or Twist. Hence, despite marked differences in morphology, the metastasis-derived cell lines PC3 and DU145 both exhibited an intermediate phenotype with co-expression of epithelial and mesenchymal markers.

Consistent with what was observed with the metastatic cell lines (DU145 and PC3) in this study, a large study conducted by Smith and colleagues (1979), on several cell lines derived from non-malignant tissues, primary carcinomas and metastatic carcinomas, demonstrated that cell lines derived from metastatic tumours displayed very little or no fibronectin expression.

Despite being derived from a primary tumour, the EMT-associated marker profile of the P4E6 cell line was analogous to that of the PC3 cells. Hence, this cell line also exhibited an overall intermediate phenotype with no clear distinction between epithelial and mesenchymal populations.

Contrary to the PC3, DU145 and P4E6 cell lines, which comprised a majority of cells expressing vimentin, the OPCT-2 cell line possessed few vimentin-positive cells. However, the vimentin-positive OPCT-2 cells co-expressed epithelial markers hence, did not represent a pure mesenchymal population. The production of fibronectin by this cell line may suggest the presence of mesenchymal-like cells. However, this was not sufficient evidence as epithelial cells have also been shown to produce fibronectin (Smith et al., 1979). Furthermore, this cell line did not appear to express any of the three EMT-associated transcription factors or the mesenchymal CAM, N-Cadherin. Therefore, the OPCT-2 cell line may have contained a population of cells which had only partially transitioned via EMT.

Microscopic examination of OPCT-1 cells revealed the presence of morphologically distinct populations, some of which resided in colonies with cobblestone morphology, while the other cell types demonstrated fibroblastoid/mesenchymal morphologies and were situated in between the colonies of epithelial-looking cells. This observation was verified using immunofluorescence, which revealed that the OPCT-1 cell line was unique in its possession of distinct epithelial and mesenchymal populations as evidenced by single-positive E-Cadherin and vimentin populations. As anticipated, the colonies exhibiting cobblestone morphology showed high staining intensities for E-Cadherin and cytokeratins.
whereas, the fibroblastoid cells frequently demonstrated single vimentin-positivity. However, co-expression of cytokeratins was also observed among some of the vimentin-positive cells. Dual-expression of vimentin and cytokeratins is not an uncommon finding in cancer cells and this observation supports the notion that the Type 3 EMT process, activated in cancer, may be incomplete (Leong et al., 1988; Hollier et al., 2009). Alternatively, these cells may have been in the process of transitioning via EMT. Interestingly, the vimentin-positive cells in this cell line exhibited sophisticated vimentin expression and the majority of these cells co-expressed fibronectin. Nuclear accumulation of β-Catenin was also observed in OPCT-1 cells, however, as with the DU145 cell line, this finding must be verified using confocal microscopy. The OPCT-1 and DU145 cell lines were unique in their expression of Snail and Slug. However, while these transcription factors appeared to be expressed by the majority of cells in the DU145 cell line, fewer cells expressing Snail and Slug were observed in the OPCT-1 cell line.

Immunofluorescent screening with a panel of several EMT-associated markers identified OPCT-1 as a model cell line for the investigation of EMT as, with the exception of Twist, this cell line expressed all of the EMT-associated markers examined. Furthermore, unlike the other cell lines examined, a clear distinction between epithelial and mesenchymal populations was evident in the OPCT-1 cell line.

Since carcinomas are cancers of epithelial cells, the presence of mesenchymal-like cells in the prostate carcinoma cell line OPCT-1 appeared to be indicative of EMT. However, mesenchymal populations may also present in carcinoma cell lines as a result of stromal contamination, hence, the OPCT-1 cell line may have consisted of immortalised epithelial and stromal cells (Dollner et al., 2004). In order to address this concern, and verify that the mesenchymal populations had arisen from epithelial cells, the OPCT-1 cell line was cloned by limiting dilution and 51 of the clones, derived using this technique, were screened by immunofluorescence for the expression of E-Cadherin and vimentin. In addition, the 51 OPCT-1 clones were dual-stained with CD24 and CD44, to address the cancer stem cell phenotype (CD44+/CD24-) reported for several human malignancies, including prostate cancer (Hurt et al., 2008). However, since all of the clones expressed CD44, and the CD24 staining patterns were ambiguous, this screening did not provide convincing evidence for the presence of putative prostate cancer stem cells.
The results of the immunofluorescence screening of the 51 OPCT-1 clones revealed that they comprised mixed populations of E-Cadherin/vimentin-expressing cells. Furthermore, distinct morphological differences were frequently observed between the single E-Cadherin-positive and single vimentin-positive populations, within the clones. Therefore, clonally derived OPCT-1 populations appeared to comprise a mixture of epithelial and mesenchymal cell types which had not arisen as a result of stromal contamination and rather, may have arisen via EMT.

Interestingly, the clones varied considerably with respect to the abundance of vimentin-positive cells. In addition, some clones presented with single vimentin-positive, fibroblastoid populations whereas others presented with dual E-Cadherin/vimentin-positive cells that did not exhibit the fibroblastoid morphology typical of mesenchymal cells. Variation in vimentin-positivity among the OPCT-1 clones implied that these cells differed in their ability and frequency to execute the EMT programme. Furthermore, since vimentin is the intermediate filament expressed by primitive cell types, the co-expression of E-Cadherin and vimentin by prostate cancer cells exhibiting epithelial morphology may have provided evidence for a population of primitive (i.e. stem-like) cells in the OPCT-1 clones.

This work demonstrated heterogeneity among clones derived from the same cell line. Therefore, these clones provided a model which could be exploited for the investigation of EMT in human prostate cancer cells, without the need for artificial induction of this phenomenon via ectopic expression of EMT-inducing transcription factors or stimulation with growth factors such as TGF-β (Mani et al., 2008; Kong et al., 2010; Li and Zhoul, 2011; Moustakas and Heldin, 2012).

In order to select appropriate clones for further investigation, 12 clones with differing proportions of vimentin-positive cells were selected and re-screened for the expression of E-Cadherin and vimentin by IF. Repeated immunofluorescent screening, following freezing, thawing and passaging of the 12 clones in culture, revealed that most of the clones maintained a constant phenotype with respect to vimentin-positivity. Interestingly, however, the two clones in which vimentin-positive cells had not previously been identified, demonstrated vimentin-positivity in a minute population of cells, which were only identified during the third IF screening. Therefore, none of the 51 OPCT-1 clones proved to be vimentin-negative.
Following the three IF screenings of the 12 OPCT-1 clones of interest, five OPCT-1 clones, which exhibited distinct and stable growth characteristics and ranged from very low to very high in terms of vimentin-positivity, were selected for further interrogation (Chapters 5 and 6).

In summary, the work reported in this study identified OPCT-1 as a suitable prostate cancer cell line for the investigation of epithelial to mesenchymal transition. In addition, phenotypically distinct OPCT-1 clones were derived and selected for further investigation using *in vitro* and *in vivo* studies to address a possible correlation between EMT and a stem-like signature in human prostate cancer cells.
Chapter 5:

Investigating the Expression of EMT and CSC-Associated Proteins by the OPCT-1 Clones – is there a Link between the EMT and CSC Marker Profiles of Human Prostate Cancer Cells?

5.1 Introduction

Epithelial to mesenchymal transition has been studied extensively in the context of breast cancer however, although evidence is emerging, the significance of this phenomenon and its contribution to the generation of prostate cancer stem cells remains to be shown. The derivation and selection of five OPCT-1 clones, which ranged from very low to high in terms of vimentin expression (Chapter 4), provided a model on which further studies on epithelial to mesenchymal transition in human prostate cancer cells could be conducted.

Most recent studies, implicating EMT in the generation of cancer stem cells, interrogated cells in which EMT was artificially induced either via ectopic expression of transcription factors including Snail and Twist, stimulation with growth factors such as TGF-β and EGF, or overexpression of growth factors such as PDGF-D (Mani et al., 2008; Kong et al., 2010; Li and Zhoul, 2011; Moustakas and Heldin, 2012). However, this study was conducted on a range of phenotypically distinct, clonally-derived cells, some of which transitioned spontaneously in culture.

The initial interrogation of the five OPCT-1 clones, documented in this chapter, involved re-cloning the clones to confirm EMT, quantifying the percentage of vimentin-positive cells in each clone and screening the clones for a panel of EMT and CSC-associated
markers by immunofluorescence and Western blotting. These studies were conducted in order to phenotype the clones with regards to their EMT and CSC status’, prior to conducting further in vitro and in vivo characterisation assays (Chapter 6).

5.2 Results

Throughout this study, parental OPCT-1 cells were assessed in parallel with the clones. It is however, important to note that differences observed in the results obtained for parental OPCT-1 between tests and repeats were to be expected. This is due to the fact that, unlike the clones, this cell line was not clonally derived and constituted a mixed population of cells with different properties, the proportions of which were subject to change during culture and, in turn, could influence the outcome of the assays. In order to limit the variation between tests, throughout the duration of this study the clones and parental OPCT-1 were assayed within a range of four passages. Furthermore, IF screening of the clones in parallel to each assay confirmed that they maintained the proportions of E-Cadherin and vimentin-positive cells which were originally observed (data not shown).

For ease of interpretation, unless otherwise stated, throughout the duration of this work the clones will be represented in order of increasing vimentin positivity; P5B3, P6D4, P5F3, P2B9, P4B6.

5.2.1 Confirmation of EMT and Determination of Cloning Efficiency by Re-cloning

Prior to conducting characterisation assays on the five OPCT-1 clones of interest, it was necessary to re-confirm that clonally derived populations of OPCT-1 cells did, in fact, consist of a mixture of epithelial and mesenchymal cells. This was necessary as there was a chance that the procedures which followed the cloning by limiting dilution assay, namely; expansion, freezing and immunofluorescence screening of the clones, may have introduced contamination across different clones. Hence, this could lead to false evidence of transitioning cells. To address this possibility, each of the five OPCT-1 clones and parental OPCT-1 cells were seeded at single-cell density, 32 wells per clone, into fluorescence compatible 96-well plates using a MoFlo™ XDP High-Speed Cell Sorter.
After a period of growth, the wells were examined to confirm the presence of only one colony per well prior to counting, fixing and staining the colonies by immunofluorescence using antibodies directed against E-Cadherin and vimentin. This assay ensured that minimal handling of the (re)cloned cells was necessary prior to conducting immunofluorescence directly in the wells in which they grew. Therefore, it was fair to assume that any mixed populations occurred as a result of transitioning cells, rather than contamination with other clones.

Figure 5.1 demonstrates that mixed populations of cells expressing both epithelial and mesenchymal markers arose in clonally derived populations of OPCT-1 cells. In fact, all of the five OPCT-1 clones generated colonies with E-Cadherin and vimentin expression. Of a total of 96 wells seeded (across the three assays), clone P5B3, which contained a very small population of vimentin-positive cells, formed only one colony with vimentin-positive cells. Interestingly, with the exception of clone P4B6, all clones and parental OPCT-1 generated colonies of cells which were only positive for E-Cadherin. Remarkably, clone P4B6 also generated loose fibroblastoid colonies which only expressed vimentin. This was of particular interest as, until this observation, it remained to be shown in this study whether the vimentin-positive cells were capable of spawning more vimentin-positive cells. Also of interest is the fact that some of the clones and parental OPCT-1 cells formed colonies with a number of cells exhibiting normal E-Cadherin expression – with accumulation of this protein in the junctions between cells (examples indicated by orange arrows).

The localisation of the vimentin-positive cells, predominantly on the edge of the colonies, alluded to the fact that these had arisen as a result of epithelial cells transitioning via EMT. Furthermore, it was evident in some of the wells that the vimentin-positive cells were beginning to migrate away from the original colony (indicated by the white arrow). Therefore, this experiment provided an insight to the metastatic process.
**Figure 5.1:** Dual immunofluorescent staining of re-cloned clones P5B3, P6D4, P5F3, P2B9, P4B6 and parental OPCT-1 in fluorescence-compatible 96 well plates using a murine monoclonal antibody against the epithelial marker, E-Cadherin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. E-Cadherin was labelled with an anti-mouse Alexa Fluor®568-conjugated secondary antibody and vimentin was labelled with an anti-rabbit Alexa Fluor®488-conjugated secondary antibody. Nuclear staining was achieved using mounting media with DAPI. (n=3). A-E represent separate wells from across the 3 assays. Representative images. Image magnification at x20. Orange arrows indicate cells with membranous E-Cadherin expression. White arrow identifies vimentin-positive cells which appeared to have migrated away from the original colony.
Prior to conducting IF on the re-cloned clones, the colonies were counted in order to determine if statistically significant differences in the number of colonies formed by the clones and parental OPCT-1 were observed. Figure 5.2 illustrates the number of colonies formed by each of the clones and parental OPCT-1 out of the 32 wells seeded (1 cell per well) in three separate assays.

![Re-cloning of Clones](image)

**Figure 5.2:** Demonstrating the number of colonies formed by each of the clones and parental OPCT-1 following cell sorting at single-cell density into 32 wells per cell line in three separate assays. (n=3) Data are represented as the mean number of colonies ± SEM.

Overall, there was no statistical significance in the number of colonies formed by the clones per 32 wells. On average, clones P6D4 and P4B6 formed the most colonies as more than half of the cells seeded (>16) formed colonies in all three assays whereas clone P2B9 formed the fewest colonies as less than half of the cells seeded formed colonies in all three assays.

From this assay, the mean cloning efficiency of each of the clones and parental OPCT-1 was calculated in order to explore a possible connection between an increase in vimentin positivity (i.e. EMT) and enhanced cloning efficiency. Cloning efficiency relates to the percentage of (seeded) wells that grow a colony of cells from a single cell. Hence, reflects the ability of a particular cell type to be cloned (i.e. form colonies from single cells).
The mean cloning efficiencies were calculated by dividing the average number of colonies which grew for each cell type by the number of wells seeded (32) and multiplying by 100. The results for each clone and parental OPCT-1 are displayed in Table 5.1.

Table 5.1: Displaying the mean cloning efficiencies of parental OPCT-1 and five clones derived from this cell line

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mean Cloning efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>41%</td>
</tr>
<tr>
<td>P5B3</td>
<td>42%</td>
</tr>
<tr>
<td>P6D4</td>
<td>61%</td>
</tr>
<tr>
<td>P5F3</td>
<td>58%</td>
</tr>
<tr>
<td>P2B9</td>
<td>33%</td>
</tr>
<tr>
<td>P4B6</td>
<td>61%</td>
</tr>
</tbody>
</table>

Clones P6D4 and P4B6 showed the highest cloning efficiencies, even higher than that of the parental OPCT-1 cell line, whereas, clone P2B9 demonstrated the lowest cloning efficiency (Table 5.1). These results confirmed that cloning efficiency did not increase concomitantly with vimentin positivity. Therefore, in this experiment, EMT of prostate cancer cells did not confer enhanced cloning efficiency.
5.2.2 Quantification of Vimentin-Positive Populations

After having confirmed that the OPCT-1 clones were indeed showing evidence of EMT, flow cytometry was utilised to quantify the percentage of vimentin-positive cells within each clone. This could serve as an indirect measure of the amount of EMT occurring in the clones, however it is important to consider that vimentin is also the intermediate filament expressed by naive epithelial cells hence, in theory, vimentin could also identify a cancer stem/progenitor cell population (Azumi et al., 1987; Korsching et al., 2005).

Figure 5.3 demonstrates example flow cytometric data to exemplify how the percentage of vimentin-positive cells was determined and a graph to illustrate the variation in the percentage of vimentin-positive cells present among the clones.

Figure 5.3: Demonstrating sample flow cytometric data (A) from which the percentage of vimentin-positive cells present in each of the clones and parental OPCT-1 was determined \((p < 0.0019, \text{Friedman statistic <19})\) (B). Sample data are shown for the least vimentin-positive clone; P5B3 and the most vimentin-positive clone; P4B6. Flow cytometry was performed using a PE-conjugated isotype control to gate the cells and a PE-conjugated anti-vimentin antibody to identify the population of cells expressing vimentin. The positive populations were defined as cells present in gate B (vimentin-PE) and the percentage of positive cells present in each clone was calculated by subtracting the percentage of cells in gate A (iso-PE) from the percentage in gate B (vimentin-PE). Representative data. \((n=4)\).
The results depicted in Figure 5.3 (B) support the observations made by immunofluorescence and the rank order of the clones with regards to vimentin-positivity remained as; P5B3, P6D4, P5F3, P2B9, P4B6. Furthermore, the differences in vimentin expression between the clones were statistically significant (p < 0.0019, Friedman statistic < 19).

The mean percentages of vimentin-positive cells present in each clone and parental OPCT-1 were calculated from the four independent flow cytometry experiments, conducted as described in Figure 5.3, and are presented in Table 5.2.

Table 5.2: Displaying the average percentage of vimentin-positive cells present in parental OPCT-1 and five clones derived from this cell line

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mean % Vimentin⁺ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>1.07%</td>
</tr>
<tr>
<td>P5B3</td>
<td>0.33%</td>
</tr>
<tr>
<td>P6D4</td>
<td>0.51%</td>
</tr>
<tr>
<td>P5F3</td>
<td>1.29%</td>
</tr>
<tr>
<td>P2B9</td>
<td>5.72%</td>
</tr>
<tr>
<td>P4B6</td>
<td>29.86%</td>
</tr>
</tbody>
</table>

Almost a third of P4B6 cells expressed vimentin (Table 5.2). This clone was by far the most positive for the expression of vimentin, almost 100 times more positive than clone P5B3 and, with the exception of clone P2B9, more than 10 times more positive than the other clones and parental OPCT-1 cells. Clone P2B9 was the second most vimentin-positive clone with approximately five times fewer vimentin-positive cells than clone P4B6.
5.2.3 Investigating the Expression of EMT and CSC-Associated Markers by Immunofluorescence

As with the five prostate cancer cell lines screened in Chapter 4, the OPCT-1 clones were screened by immunofluorescence for a panel of several EMT-associated markers. In addition to these, they were also screened for the prostate cancer stem cell-associated markers; CD44 and integrin α2β1 and the three transcription factors critical for the maintenance of self-renewal and pluripotency in embryonic stem cells; Nanog, Oct4 and Sox2.

Figures 5.4 to 5.12 demonstrate the results to the immunofluorescent screening of the clones, please refer to Appendix V for the appropriate positive and negative controls thereof.

The clones were initially selected and subsequently re-screened based on their expression of the epithelial marker E-Cadherin and the mesenchymal marker vimentin. In order to verify the existence of epithelial populations using an additional epithelial marker, they were screened for the expression of cytokeratins (CK) in conjunction with vimentin. All five of the clones comprised cells which expressed cytokeratins (Figure 5.4). Surprisingly, clone P5B3 appeared to be the least positive for cytokeratins as there were several CK-negative cells present in this clone however, as they were also negative for the expression of vimentin, the identity of these dual-negative cells remained elusive. All other clones comprised a mixture of cells, predominantly expressing only cytokeratins, but some co-expressed cytokeratins and vimentin. It is important to note that, although not represented in this figure, single vimentin-positive cells were observed in clones P2B9 and P4B6.

As the expression of nuclear β-Catenin has been associated with induction of EMT, the expression of this protein was examined in the clones to investigate a possible correlation between an increase in vimentin positivity and β-Catenin accumulation in the nucleus. However, Figure 5.5 demonstrates that, although membranous and cytoplasmic β-Catenin expression was apparent in all five clones, without assessment by confocal microscopy it was difficult to ascertain whether nuclear staining was present in any of the cells. Interestingly, cells which were strongly positive for vimentin expression in clones P5F3, P2B9 and P4B6 appeared to express less β-Catenin than cells which did not express this intermediate filament.
Figure 5.4: Dual immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a murine pan polyclonal antibody against the epithelial marker, cytokeratin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. Secondary antibody labelling and nuclear staining were achieved as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Figure 5.5: Dual immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a murine monoclonal antibody against β-Catenin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. Secondary antibody labelling and nuclear staining were achieved as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
In order to ascertain whether the vimentin-positive cells in the clones were producing cellular fibronectin, another marker associated with mesenchymal cells, the clones were screened with fibronectin and vimentin antibodies (Figure 5.6). As anticipated, the expression of fibronectin increased concomitantly with vimentin expression. Interestingly, there was no fibronectin detected in clone P5B3 although a vimentin-positive cell was observed, based on the infrequency of vimentin-positive cells in this clone and the lack of fibronectin expression by these cells, the vimentin-expressing population in this clone may have represented primitive cells, rather than cells which had undergone EMT. The same may be true of clone P6D4 which also contained a small population of vimentin-positive cells which did not express fibronectin. Hence, not all of the vimentin-positive cells produced fibronectin. Furthermore, clone P6D4 demonstrated the expression of fibronectin by a vimentin-negative cell (indicated by blue arrows). Therefore, although fibronectin expression increased concurrently with vimentin expression overall, the expression of this protein was not exclusive to vimentin-positive cells. This finding is consistent with studies which have demonstrated the production and secretion of fibronectin by cultured epithelial cells (Smith et al., 1979).

N-Cadherin is another marker associated with mesenchymal cells and has been shown to be upregulated and replace E-Cadherin in the so-called “cadherin switch” during EMT of prostate cancer cells (Gravda et al., 2007). As such, the expression of this protein was expected to be highest in the clones with the largest populations of vimentin-positive cells. Figure 5.7 demonstrates that N-Cadherin expression was observed in all of the clones with the exception of clone P5B3. As anticipated, the highest levels of this protein were observed in the clones with the highest percentage of vimentin-positive cells; clones P2B9 and P4B6. Clone P4B6 exhibited intense staining in the cells with fibroblastoid morphology however the staining appeared to be more intense in the colonies of cells in clone P2B9. Please note that the apparent staining in clone P5B3 was due to non-specific staining of dividing cells.
**Figure 5.6: Dual immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a murine monoclonal antibody against cellular fibronectin and a rabbit polyclonal antibody against the mesenchymal marker, vimentin. Secondary antibody labelling and nuclear staining were achieved as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20. Blue arrows indicate fibronectin expression by vimentin-negative cells.**
Figure 5.7: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a murine monoclonal antibody against N-Cadherin. Secondary antibody labelling was achieved using an Alexa Fluor®588-conjugated anti-mouse antibody. Nuclear staining was achieved as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Snail, Slug and Twist are three of several zinc finger transcription factors which are known to suppress E-Cadherin expression and induce EMT. The expression of these transcription factors was investigated in the OPCT-1 clones in an effort to provide further evidence to support the hypothesis that the vimentin-high clones were undergoing EMT.

As can be observed in Figure 5.8, Snail was detected in all of the clones. The staining of the clones with the Snail antibody achieved similar patterns to those previously observed in parental OPCT-1 and DU145, in that Snail was localised in the cytoplasm with a dotty appearance. Clones P5F3 and P4B6 demonstrated the highest staining intensities and clones P5B3 and P2B9 demonstrated the lowest staining intensities for Snail. The comparatively low levels of Snail in clone P2B9 were surprising as this clone comprised the second largest population of vimentin-positive cells. However, the low levels of this transcription factor in clones P5B3 and P6D4 was consistent with the fact that these clones expressed low levels of vimentin.

Analogous to the staining patterns observed with the Snail antibody, all five clones showed evidence of Slug expression with clones P5B3 and P2B9 exhibiting very low levels and clones P5F3 and P4B6 demonstrating higher staining intensities (Figure 5.9). However, clone P6D4 appeared to express higher levels of Slug than Snail. Positive staining of clone P4B6 was predominantly observed in cells with a mesenchymal/fibroblastoid morphology.

Figure 5.10 demonstrates that none of the OPCT-1 clones stained positive for the expression of the transcription factor Twist. This was consistent with the lack of staining observed in previous studies with parental OPCT-1 however; it was hypothesised that perhaps with an enriched population of cells undergoing EMT, Twist would be observed. It is important to note that this antibody failed to detect Twist in a positive control cell line (data not shown) therefore, the efficacy of this antibody, using the IF method followed throughout this study, was questionable. However, as this antibody was capable of detecting Twist in whole cell lysates of the OPCT-1 clones and parental OPCT-1 by Western blotting (Figure 5.14), it is likely that it was unsuitable for IF and that some of the clones did express this transcription factor.

Overall, these results did not demonstrate a direct correlation between vimentin-positivity and the expression of EMT-inducing transcription factors as clone P2B9 demonstrated lower levels of Snail and Slug staining than clones P5F3 and P6D4. However, verification by dual staining with vimentin would be necessary to confirm this.
Figure 5.8: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a goat polyclonal antibody against Snail. Secondary antibody labelling was achieved using an anti-goat Alexa Fluor®568-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Figure 5.9: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a goat polyclonal antibody against Slug. Secondary antibody labelling was achieved using an anti-goat Alexa Fluor®568-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Figure 5.10: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a rabbit polyclonal antibody against Twist. Secondary antibody labelling was achieved using an anti-rabbit AlexaFluor®488-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Recently there has been a surge of interest surrounding the topic of EMT and its involvement in the generation of CSCs. Immunofluorescence using a panel of several EMT-associated markers revealed that the OPCT-1 clones provided a model for the investigation EMT of prostate cancer cells. As such, these clones enabled the examination of a possible link between EMT and the generation of prostate CSCs. In 2005, Collins et al., identified putative prostate cancer stem cells using a panel of markers which had been previously exploited to identify normal prostate stem cells (Collins et al., 2005). Therefore, as the markers for EMT and prostate cancer stem cells were well established, it was possible to exploit some of these to investigate a potential correlation between EMT and PCSCs using the OPCT-1 clones. To that end, the OPCT-1 clones were screened for two of the cell-surface proteins shown to be highly expressed by PCSCs; CD44 and integrin α2β1, in addition to three transcription factors known to play a key role in the maintenance of self-renewal and pluripotency; Nanog, Oct4 and Sox2.

Figure 5.11 illustrates that all of the OPCT-1 clones stained positive for the expression of CD44, a family of proteins shown to regulate growth, survival, differentiation and migration of cancer cells (Orian-Rousseau, 2010). Clones P5F3 and P4B6 appeared to express the highest levels of CD44 and clones P5B3 and P2B9 contained the largest number of CD44-low/negative cells among the clones (indicated by yellow arrows). Therefore, CD44 positivity did not directly correspond with vimentin positivity as the clone with the second largest population of vimentin-positive cells (P2B9) presented with several CD44-low/negative cells and did not demonstrate the second highest staining intensity overall.

Integrin α2β1 is a transmembrane receptor for extracellular matrix proteins such as collagen and laminin, adhesion molecules such as E-Cadherin and several other ligands including matrix metalloproteinase-1 (MMP-1). Among other functions, it is known to play a role in the generation and organisation extracellular matrix proteins and mediate interactions between adhesion molecules on adjacent cells (Zutter and Edelson, 2007). Figure 5.12 demonstrates variable expression of this molecule among the clones with clone P5B3 expressing the highest levels and clone P6D4 expressing the lowest levels. Clone P4B6 presented with two distinct populations with regards to the expression of this molecule, where the colonies of cells expressed integrin α2β1 and the loose, mesenchymal-like cells (indicated by yellow arrows) did not. This is of particular interest as prostate cancer stem cells were shown by Collins and colleagues to express high levels of this
receptor protein however it appears that the mesenchymal-like (EMT derived) cells were negative for the expression of this protein. Therefore, this provides evidence to support the notion that prostate cancer stem cells and EMT-derived cells are mutually exclusive. Although dual vimentin/integrin α2β1 staining was not conducted on clone P4B6, it was conducted on parental OPCT-1. Figure 5.13 demonstrates that vimentin and integrin α2β1 were differentially expressed in parental OPCT-1 cells (indicated by arrows) therefore, this figure provides evidence to suggest that EMT of prostate cancer cells does not generate the same prostate cancer stem cells as those identified by Collins et al., 2005.

As tumour development has long been compared with abnormal embryogenesis, it is hardly surprising that several studies have reported the expression of three key transcription factors involved in the maintenance of self-renewal and pluripotency of embryonic stem cells (Nanog, Oct4 and Sox2) in putative cancer stem cells (Kim et al., 2008; Gong et al., 2012; Kumar et al., 2012; Shan et al., 2012). To address whether these transcription factors were more highly expressed in clones which contained populations of cells undergoing EMT, hence draw parallels between the CSC and EMT phenotype, the OPCT-1 clones were examined by immunofluorescence for the presence of Nanog, Oct and Sox2. Using this technique, none of these transcription factors were detected in any of the OPCT-1 clones (See Appendix V). Additional immunofluorescent staining of the pluripotent embryonic carcinoma cell line, NTERA2/D1, confirmed that the antibodies used were suitable for the detection of these transcription factors by IF (See Appendix V). However, as with the Twist antibody, these antibodies were capable of detecting the corresponding proteins in whole cell lysates of the OPCT-1 clones and parental OPCT-1. The inconsistencies in the results obtained from the two techniques may be due to differences in the experimental conditions employed and will be discussed in more detail in Section 5.3.
**Figure 5.11:** Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a mouse monoclonal antibody against CD44. Secondary antibody labelling was achieved using an anti-mouse AlexaFluor®568-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20. Yellow arrows indicate areas of low CD44 expression.
Figure 5.12: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6 using a mouse monoclonal antibody against integrin α2β1. Secondary antibody labelling was achieved using an anti-mouse AlexaFluor®568-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3) Representative images. Image magnification at x20. Yellow arrows demonstrate integrin α2β1-negative cells in the P4B6 clone.
Figure 5.13 demonstrates that the EMT-derived, vimentin-positive cells present in the OPCT-1 cell line did not express integrin α2β1. This result confirmed that the EMT-derived cells must have been distinct from the putative prostate cancer stem cells (CD44^+/CD133^+/integrin α2β1^hi^) described by Collins et al., 2005 therefore, integrin α2β1 expression potentially distinguishes between the original tumour-initiating prostate cancer stem cells and the cancer stem-like cells generated by EMT.

Figure 5.13: Dual immunofluorescent staining of parental OPCT-1 using a mouse monoclonal antibody against integrin α2β1 and a rabbit polyclonal antibody against vimentin. Secondary antibody labelling and nuclear staining was performed as previously described in Figure 5.1. (n=3). Red arrows indicate integrin α2β1-positive cells, yellow arrows indicate vimentin-positive cells. Representative images. Image magnification at x20.
5.2.4 Investigating the Expression of EMT and CSC-Associated Markers by Western blotting

In order to validate and confirm the results of the IF screening, where possible, Western blotting was performed on the same markers using whole cell lysates prepared from the clones and parental OPCT-1.

Figure 5.14 reveals the protein banding patterns achieved by Western blot analysis of seven EMT-associated markers.

Western blot analysis revealed that P4B6 expressed the lowest levels of the epithelial marker E-Cadherin but the highest levels of the mesenchymal markers vimentin, N-Cadherin and EMT transcription factor Twist. Using this technique, vimentin was only detected in clones P5F3, P2B9 and P4B6, with expected increasing levels. As anticipated, clone P5B3 consistently expressed the lowest levels of vimentin, N-Cadherin and Twist.
Overall, these results were consistent with the staining patterns achieved by IF however, Snail and Slug were not detected in any of the clones using this method.

Figure 5.15 demonstrates the protein banding patterns achieved by Western blot analysis of four CSC-associated markers.

These results illustrate that clone P4B6, the most vimentin-positive clone, expressed the highest levels of CD44 and Oct4 but the lowest levels of Nanog. Consistent with IF, clone P5B3, the least vimentin-positive clone, expressed the lowest levels of CD44. Interestingly, clone P6D4 expressed the highest levels of Nanog but not the highest levels of Oct4. Using this method, Sox2 expression was only detected in parental OPCT-1, which also expressed the highest levels of Oct4. The two bands detected for Oct4 may represent the two isoforms of this transcription factor; Oct4 A and Oct4 B.

It is important to note that, while the clones with the highest and lowest protein expression remained consistent across the five repeats, the intermediate banding patterns varied. Hence, it was not possible to rank the clones in order of expression of each of the proteins and measurement by densitometry was unsuitable.
Copper staining of the nitrocellulose membrane prior to immunoprobing (not shown) and β-actin staining confirmed that equal amounts of protein were loaded into the SDS-PAGE gels.

In order to confirm the efficacy of the antibodies used to detect the CSC associated transcription factors, lysates from NTERA2/D1 cells were prepared and analysed by Western blotting. Lysates from the murine embryonic fibroblast cell line, NIH 3T3, were also used as a positive control for the expression of the EMT-associated transcription factors Snail and Slug.

Figure 5.16 demonstrates the protein banding patterns obtained from the positive control cell lines.

![Figure 5.16: Western blot analysis of CSC-associated transcription factor expression by NTERA2/D1 (A) and EMT-associated transcription factors by NIH 3T3 (B). Proteins were stained overnight using antibodies which were used in accordance with the manufacturer’s instructions. (n=2) Representative images.](image)

The Nanog, Oct4, Sox2, Snail and Slug antibodies used in this study were suitable for the detection of protein by Western blotting (Figure 5.16).

### 5.2.5 Summary of Immunofluorescence and Western Blot Analysis

Tables 5.3 and 5.4 summarise and compare the results obtained from the immunofluorescence (IF) screening and Western Blot analyses (WB) of the EMT and CSC associated marker expression of the OPCT-1 clones, respectively. Please note that the following summary tables do not distinguish between low and high protein expression, only absence or presence. Please refer to Figures 5.4 to 5.15 to observe differences in the staining intensities between the clones.
Table 5.3 Summarising the results of the IF and WB screening of the OPCT-1 clones with the EMT-associated markers

<table>
<thead>
<tr>
<th>Protein</th>
<th>E-cad</th>
<th>CK</th>
<th>B-cat</th>
<th>Vim</th>
<th>Fibro</th>
<th>N-cad</th>
<th>Snail</th>
<th>Slug</th>
<th>Twist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>IF</td>
<td>WB</td>
<td>IF</td>
<td>WB</td>
<td>IF</td>
<td>WB</td>
<td>IF</td>
<td>WB</td>
<td>IF</td>
</tr>
<tr>
<td>P5B3</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>- (+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>P6D4</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P5F3</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P2B9</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P4B6</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: - (+) denotes that vimentin positive cells were very rarely observed in clone P5B3

E-Cadherin, vimentin, fibronectin and N-Cadherin staining patterns among the clones were mostly consistent between IF and WB. However, the results obtained for Snail, Slug and Twist were inconsistent between the two methods.

Table 5.4 Summarising the results of the IF and WB screening of the OPCT-1 clones with the CSC-associated markers

<table>
<thead>
<tr>
<th>Protein</th>
<th>CD44</th>
<th>Inta2β1</th>
<th>Nanog</th>
<th>Oct4</th>
<th>Sox2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>IF</td>
<td>WB</td>
<td>IF</td>
<td>WB</td>
<td>IF</td>
</tr>
<tr>
<td>P5B3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>n/a</td>
<td>-</td>
</tr>
<tr>
<td>P6D4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
</tr>
<tr>
<td>P5F3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>-</td>
</tr>
<tr>
<td>P2B9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>-</td>
</tr>
<tr>
<td>P4B6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>-</td>
</tr>
</tbody>
</table>

The CD44 and Sox2 staining patterns observed among the clones were mostly consistent between IF and WB. However, CD44 was not detected in clone P5B3 by Western blotting but the immunofluorescence screening demonstrated clear expression of this protein by this clone. The results obtained for the transcription factors Nanog and Oct4 were inconsistent between the two methods in that neither of these proteins were detected in the clones by IF however both were detected in the clones by WB.
5.3 Discussion

This work aimed to verify that clonally derived populations of OPCT-1 cells were capable of generating mesenchymal-like cells via EMT, quantify the percentage of vimentin-positive cells in each of the five OPCT-1 clones and profile the clones with respect to the expression of selected EMT and CSC-associated markers.

The re-cloning of the five OPCT-1 clones into fluorescence-compatible 96 well plates, followed by IF staining with E-Cadherin and vimentin antibodies, confirmed that clonally derived populations gave rise to mixed cell types, as evidenced by the expression of epithelial (E-Cadherin) and mesenchymal (vimentin) markers. In fact, this assay revealed that all five of the OPCT-1 clones were capable of generating mixed populations, with regards to E-Cadherin and vimentin expression, from the single cell level. The location of the vimentin-positive cells, predominantly on the edge of the colonies, indicated that these cells had activated the EMT programme. Furthermore, the observation of isolated vimentin-positive cells, identified in colonies derived from the P4B6 clone, which appeared to have migrated away from the original colony, provided an insight into the phenomena observed in patients with metastatic disease, whereby cells at the tumour-host interface (invasive front) begin to break away, invade and migrate to distant sites (Thiery et al., 2009).

With the exception of clone P4B6, the re-cloned OPCT-1 clones also formed colonies which exclusively expressed E-Cadherin. Previous studies have demonstrated that clonally derived populations, isolated from melanoma and breast cancer cell lines differed significantly with regards to their metastatic capabilities in vivo and this may be due to differences in the EMT-status of the cells (Kozlowski et al., 1984; Uruid et al., 2002). The re-cloning experiment conducted in this study was particularly interesting, as it indicated that even within a clonally derived population, cells differed in their ability to activate the EMT programme. This variation was likely to be governed by the epigenetic nature of the changes which activate EMT in many cases (Brabletz, 2012).

Remarkably, clone P4B6 formed clonal populations which were 100% vimentin-positive/E-Cadherin-negative and, unlike the E-Cadherin-expressing colonies, these colonies consisted of loose clusters of fibroblastoid cells. Furthermore, these colonies comprised fewer cells than those with E-Cadherin expression. This observation revealed that the
cloned, EMT-derived, mesenchymal-like carcinoma cells were capable of proliferating. However, the comparatively smaller cell numbers present in the single vimentin-positive colonies provided evidence in support of the hypothesis that epithelial carcinoma cells are more proliferative than their EMT-derived, mesenchymal-like counterparts. Hence, following migration and extravasation, metastatic (mesenchymal-like) cancer cells are believed to re-differentiate into epithelial cells via mesenchymal to epithelial transition (MET), in order to form macrometastases (Kalluri and Weinberg, 2009; Foroni et al., 2012).

In order to investigate the MET programme, a clonally derived population of vimentin-positive/E-Cadherin-negative cells was established from clone P4B6. Preliminary observations revealed that this clone appeared to proliferate at a slower rate than the other OPCT-1 clones in culture (data not shown) however, further in vitro and in vivo studies will be conducted to address whether or not these cells are capable of differentiating into epithelial cells. This work will be of great interest because, although activation of MET by mesenchymal-like carcinoma cells has been hypothesised to be necessary for macrometastasis formation, the role of MET in cancer progression remains to be conclusively demonstrated (Brabletz, 2012).

The re-cloning assays revealed that clones P6D4, the second least vimentin-positive clone, and P4B6, the most vimentin-positive clone, possessed the joint-highest cloning efficiencies and clone P2B9, the second most vimentin-positive clone, demonstrated the lowest cloning efficiency. However, due to large variation in the number of colonies formed by clone P5F3 across the three repeats, these results were not statistically significant overall. Nonetheless, the data obtained demonstrated that cloning efficiency did not correlate with vimentin-positivity.

Using flow cytometry, this study determined that the order of vimentin-positivity among the clones was in keeping with what was previously observed by IF. Furthermore, the variation in vimentin-positivity across the clones was shown to be statistically significant.

The results obtained from the IF and WB analyses were mostly as expected in that clone P5B3, the least vimentin-positive clone, had an epithelial signature with low expression of the mesenchymal markers (vimentin), fibronectin and N-Cadherin and high expression of the epithelial markers E-Cadherin and cytokeratins and clone P4B6, the most vimentin-positive clone, not only demonstrated the highest levels of the mesenchymal markers,
(vimentin) and N-Cadherin, but also had the lowest expression of the epithelial marker, E-Cadherin. However, clone P4B6 also expressed high levels of epithelial cytokeratins, thus, this clone exhibited a mixed signature with the highest proportion of mesenchymal-like cells of the clones.

With regards to clone P4B6, the IF and WB screenings appeared to demonstrate that cells with an EMT signature also demonstrated a CSC signature. This is because, as well as exhibiting high mesenchymal-marker expression, this clone also expressed the highest levels of the CSC-associated proteins CD44 and Oct4. Oct4 expression in cancer cells is frequently implied to mark putative cancer stem cells (Jeter et al., 2009). Furthermore, Oct4 has also been demonstrated to be an important mediator of migration and invasion (Chang et al., 2008). However, the expression of Oct4 by P4B6 was not enough to confirm that this clone possessed stem-like attributes and this hypothesis required verification in future sphere-forming, clonogenic, proliferation, tumourigenesis and invasion assays (Chapter 6). Interestingly, clone P4B6 demonstrated low levels of the stem cell-associated transcription factors Nanog and Sox2, which was inconsistent with findings by others that have demonstrated the co-expression of Nanog, Oct4 and Sox2 in glioma cancer stem cells (Guo et al., 2011).

Surprisingly, clone P6D4 expressed the highest levels of Nanog, but not the highest levels of Oct4 and Sox2 (detected by Western blotting). Furthermore, the prostate cancer stem cell marker, integrin α2β1 was not detected in this clone by IF. Recently, Jeter et al., 2011, revealed that Nanog promoted cancer stem cell characteristics, therapeutic resistance, CD133 and ALDH upregulation and prostate cancer resistance to androgen deprivation. Therefore, despite comprising very few vimentin-positive cells and failing to express one of the putative prostate cancer stem cell markers, this clone may be enriched for cancer stem cells hence, may be expected exhibit an aggressive stem-like phenotype. This concept is re-visited in more detail in Chapter 6 where the potential stem-like properties of the OPCT-1 clones are thoroughly investigated.

The second most vimentin-positive clone, P2B9, did not demonstrate the same staining intensities as clone P4B6 in that it expressed higher levels of E-Cadherin and fibronectin and lower levels of CD44 and Oct4. Therefore, despite expressing the second highest levels of vimentin, this clone did not appear to exhibit a stem-like signature.
As anticipated, clone P5F3 represented an intermediate phenotype with neither the highest nor lowest expression of any of the proteins examined.

Several inconsistencies were observed between the IF and WB results. The CD44 and Sox2 staining patterns observed among the clones were mostly consistent between IF and WB, however, CD44 expression was not detected in clone P5B3 by Western blotting although the IF screening demonstrated clear expression of the CD44 protein by this clone. In addition, the results obtained for the transcription factors Nanog, Oct4 and Twist were inconsistent between the two methods in that none of these proteins were detected in the clones by IF however, all three were detected in the clones by WB. Conversely, Snail and Slug expression were detected by IF but not Western blotting.

The inconsistencies observed between the IF and WB results were likely to have occurred due to differences in the procedures. Immunofluorescence detects proteins in their native form, whereas Western blotting involves a denaturation step which may interfere with antibody binding by damaging the epitopes, thereby rendering the antibody unable to bind to the protein during Western blotting. Conversely, protein denaturation may assist with antibody binding by exposing epitopes which may otherwise be concealed, accounting for positive WB results but negative IF results. In addition, the transcription factors examined in this study are known to be transiently expressed in only a small population of cells hence, Western blotting may not be sufficiently sensitive for the detection of these proteins. The inability to detect Snail by WB may be accounted for by the fact that this protein is extremely unstable, with a half-life of only 20-30 minutes in most cancer cell lines (Garcia de Herreros and Baulida, 2012).

Interestingly, Maitland et al., 2011, reported that they had observed high levels of vimentin in their primary cultures of high-grade prostate cancer cells. However, the present study provided evidence to support the notion that the prostate cancer stem cells reported by Collins et al., 2005, and EMT-derived cancer stem-like cells are distinct. This is because the EMT-derived vimentin-positive, fibroblastoid cells in the parental OPCT-1 cell line did not express the putative PCSC marker integrin α2β1. Therefore, perhaps integrin α2β1-negativity could prove useful for the identification of EMT-derived mesenchymal cells in clinical specimens. However, one previous study contradicted this finding as it demonstrated that an increase in integrin α2β1 coincided with collagen/FGF-1 induced EMT (Valles et al., 1996). Therefore, an extensive investigation into the expression of
integrin α2β1 in EMT-derived populations would prove to be an interesting avenue of research.

In summary, the five OPCT-1 clones of interest proved to be capable of generating epithelial and mesenchymal populations from a single cell, thus, showed evidence of EMT. Moreover, flow cytometric analysis, which determined the percentage of vimentin-positive cells present in each clone, corresponded with previous observations by immunofluorescence. The five OPCT-1 clones demonstrated distinct phenotypes with regards to the expression of several EMT and CSC-associated proteins. Therefore, these clones provided a suitable model for investigating the properties of cells with and without an EMT signature. The present work revealed that an EMT-signature did not always correspond with a stem-like profile (clone P2B9) and conversely, vimentin-low cells may demonstrate expression of stem cell-associated markers (clone P6D4). However, these findings must be supported with in vitro and in vivo cancer stem-cell characterisation and tumourigenesis assays. Therefore, further interrogation of the five OPCT-1 clones is required in order to conclusively ascertain or disprove a link between epithelial to mesenchymal transition and cancer stem-like cells in human prostate cancer.
Chapter 6:

Functional Assessment of the OPCT-1 Clones – is there a Link between Epithelial to Mesenchymal Transition and an Aggressive Cancer Stem Cell Phenotype in Human Prostate Cancer?

6.1 Introduction

Having profiled the OPCT-1 clones with regards to the expression of several EMT and CSC-associated markers (Chapter 5), it was then necessary to examine their biological characteristics and determine whether the expression of EMT-associated markers corresponded with properties attributed to an aggressive, migratory, cancer stem cell-like phenotype.

As previously described (Section 1.56), in order to satisfy the conditions of a cancer stem cell, several criteria must be tested, exploiting the specific growth characteristics unique to cancer stem cells. Therefore, to investigate a possible correlation between epithelial to mesenchymal transition and the acquisition of stem cell traits, the five OPCT-1 clones were subjected to several stem cell assays. Furthermore, since cancer stem cells and EMT-derived cells have both been implicated in metastasis, the invasive and migratory capacity of the five clones was also investigated. In addition, to ascertain a possible relationship between EMT and drug resistance, the drug sensitivity of the clones was examined. The quintessential property of cancer stem cells is their tumour-initiating capacity. Therefore, to address whether cells with an EMT-signature possess enhanced tumourigenic capabilities, *in vivo* tumourigenesis assays were also conducted.
6.2 Results

6.2.1 Proliferation Assays

Among other properties, cancer stem cells have been shown to possess a marked capacity for proliferation (Clevers, 2011). In order to ascertain a possible link between an EMT phenotype and proliferation, the proliferation of the OPCT-1 clones compared with parental OPCT-1 was investigated using three independent proliferation assays, the ViaLight® Plus assay, the Thymidine incorporation assay and the MTT assay (Figure 6.1).

![Figure 6.1: ViaLight® Plus (p < 0.0001, Friedman statistic < 38.27) (A), Thymidine (p < 0.0001, Friedman statistic < 51) (B) and MTT Proliferation (p < 0.0001, Friedman statistic < 47.62) (C) assays conducted on clones P5B3, P6D4, P5F3, P2B9, P4B6 and parental OPCT-1 cells. (n=3)](image)

The OPCT-1 clones and parental OPCT-1 exhibited significant differences in proliferation as evidenced by three separate assays, statistically analysed using a non-parametric, Friedman ANOVA (p < 0.0001 for all assays). Despite variation in the methods of measuring proliferation, the data obtained from the ViaLight® Plus (A) and Thymidine (B) assays were similar. These two assays clearly demonstrated that clone P4B6 was the most proliferative and clones P2B9 and P5B3 were the least proliferative. Furthermore, the results obtained from The ViaLight® Plus (A) and Thymidine (B) assays corresponded with the degree of confluency microscopically observed during cell culture, in that clone P4B6 was consistently the first clone to reach full confluence and clones P5B3 and P2B9 were consistently the last to reach full confluence.

The results obtained using the MTT assay (C) were the least consistent with what was observed microscopically during cell culture as this assay showed little variation between the cells with the exception of clone P4B6. As such, this assay was not utilised in further
Although the results to this assay were highly significant when analysed using a non-parametric, Friedman ANOVA, this analysis was likely to have been influenced by the large number of technical replicates as well as the large difference between clone P4B6 and the rest of the cells.

### 6.2.2 Investigation of Drug Resistance

Drug resistance is a property attributed to cancer stem cells and recent studies have shown that mesenchymal-like cancer cells, generated via EMT, can demonstrate resistance to chemotherapeutic agents (Huang et al., 2006; Singh and Settleman, 2010; Wang et al., 2010). Therefore, the chemotherapeutic sensitivity of the five OPCT-1 clones was investigated.

Docetaxel therapy is the “standard of care” for patients with metastatic, castrate-resistant prostate cancer (Bracarda et al., 2011). Therefore, this drug was selected for studies with the OPCT-1 clones. Prior to conducting drug assays on the clones, it was necessary to determine a suitable drug dose to apply. This was achieved by incubating parental OPCT-1 cells with a wide range of docetaxel concentrations in order to determine a suitable range, followed by dose-response ViaLight® Plus and Thymidine proliferation assays which generated a curve from which the IC50 of parental OPCT-1 could be determined for each assay. The IC50 is the half maximal inhibitory concentration of a substance and is a quantitative measure of how much of a substance is required to inhibit a biological process (in this case proliferation) by 50% (Cheng and Prusoff, 1973).

The docetaxel IC50 doses of parental OPCT-1 were successfully calculated using two independent assays (Figure 6.2). Using the ViaLight® Plus assay the IC50 of docetaxel on parental OPCT-1 was calculated as 11.15 nM. The docetaxel IC50 value of parental OPCT-1 determined using the Thymidine assay was approximately half the dose of the IC50 determined using ViaLight® Plus assay; 5.617 nM.

Due to the unsatisfactory dose response curve obtained using the ViaLight® Plus proliferation assay (Figure 6.2B), the Thymidine assay was selected for further drug assays.
Figure 6.2: Dose response curve and IC50 determination of docetaxel on parental OPCT-1 using the ViaLight® Plus (A) and Thymidine (B) proliferation assays. IC50 values were calculated using GraphPad Prism software. (n=3)
After determining the docetaxel IC50 doses for parental OPCT-1, drug assays on the five OPCT-1 clones were conducted. The OPCT-1 clones and parental OPCT-1 were subjected to treatment with the determined IC50 dose (5.5 nM), double the IC50 dose (11 nM) and media alone, prior to assessing proliferation using the Thymidine assay (Figure 6.3).

Significant differences in the proliferation rates of the clones, in response to different docetaxel treatments, were observed ($p < 0.014715$, $F < 2.318$) (Figure 6.3). Statistical analysis was performed by means of a Factorial ANOVA using STATISTICA software. The mean inhibitory effect of each drug dose on proliferation was calculated as a percentage and is presented in Table 6.1.

This assay revealed that clone P6D4 was the most resistant, only 10% inhibition with the IC50 dose and 14% inhibition with double the IC50 dose, and clone P5B3 was the most sensitive to treatment with docetaxel, 41% inhibition with the IC50 dose and 60% inhibition with double the IC50 dose. Clones P6D4, P5F3 and P4B6 were more resistant to docetaxel treatment than parental OPCT-1 and clones P5B3 and P2B9 were more sensitive to this drug than parental OPCT-1.

Therefore, according to the results obtained, drug resistance did not necessarily correspond with vimentin-positivity.
Figure 6.3: Demonstrating the proliferation of the OPCT-1 clones and parental OPCT-1 treated with control media, the docetaxel IC50 dose (5.5 nM) and double the docetaxel IC50 dose (11 nM) of parental OPCT-1. Assessed using the Thymidine proliferation assay. Data were analysed by means of a Factorial ANOVA using STATISTICA software ($p < 0.014715, F < 2.318$). (n=5)

Table 6.1 Displaying the mean percentages of docetaxel inhibition on the proliferation of the five OPCT-1 clones and parental OPCT-1.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percentage Inhibition of Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.5 nM</td>
</tr>
<tr>
<td>Parental</td>
<td>37%</td>
</tr>
<tr>
<td>P5B3</td>
<td>41%</td>
</tr>
<tr>
<td>P6D4</td>
<td>10%</td>
</tr>
<tr>
<td>P5F3</td>
<td>9%</td>
</tr>
<tr>
<td>P2B9</td>
<td>35%</td>
</tr>
<tr>
<td>P4B6</td>
<td>27%</td>
</tr>
</tbody>
</table>
6.2.3 Assessment of Clonogenicity, Sphere-formation and Self-renewal

6.2.3.1 Clonogenic Assay

Cancer stem cells demonstrate increased clonogenic capacity compared with other cancer cells. Therefore, clonogenic assays have been used to identify populations of cancer cells with stem-like characteristics (Kong et al., 2010). During this study, the clonogenicity of the five OPCT-1 clones and parental OPCT-1 cells was determined using an assay whereby cells were seeded at low (clonal) density and allowed to grow prior to counting the number of colonies. The cells which formed the largest number of colonies were deemed the most clonogenic.

Highly significant differences were observed in the clonogenic abilities of the clones, statistically analysed using a non-parametric, Friedman ANOVA (p < 0.0003, Friedman statistic < 23.66) (Figure 6.4). Clone P4B6 and clone P5B3 possessed the highest and lowest clonogenicities, respectively. This assay confirmed that the two most vimentin-positive clones, clone P4B6 and clone P2B9, were also the most clonogenic. Hence, this assay appeared to associate EMT with increased clonogenicity. However, the colonies formed by these clones were very distinct as clone P2B9 formed small colonies with few cells and clone P4B6 formed large, diverse colonies of many cells. Overall, clonogenicity did not increase with vimentin positivity, in a stepwise manner.

Figure 6.4: Demonstrating the results of the Clonogenic Assay performed on clones P5B3, P6D4, P5F3, P2B9, P4B6 and parental OPCT-1 and example images of the colonies which grew in the 6 well plates. Cells were plated at clonal density, cultured for a period of 10 days, fixed with ethanol and stained with crystal violet prior to enumerating the colonies (p < 0.0003, Friedman statistic < 23.66). (n=3)
6.2.3.2 Sphere-forming Assay

Sphere-forming, or anchorage independent growth, assays have been exploited in several studies as a means to identify cancer cells with stem-like properties (Collins et al., 2005; Mani et al., 2008; Kong et al., 2010; Clevers, 2011; Pastrana et al., 2011). These assays are believed to assess stemness as anchorage-independent growth and formation of spheres is a quality which is attributed only to stem and progenitor cells, the latter of which may be excluded from the counts based on size (Pastrana et al., 2011). In the present study, the ability of the clones and parental OPCT-1 to form spheres in non-adherent conditions was assessed in order to ascertain whether there was an association between sphere-forming ability and EMT.

This assay revealed that all five of the OPCT-1 clones were capable of anchorage independent growth (Figure 6.5). Furthermore, the differences observed in the sphere-forming abilities of the clones were highly statistically significant, statistically analysed using a non-parametric, Friedman ANOVA (p < 0.0001, Friedman statistic < 41.57). Clone P4B6 and clone P2B9 were the most and least sphere-forming, respectively. Therefore, although the most sphere-forming clone was the most vimentin-positive (P4B6), the second most vimentin-positive clone was the least capable of sphere-formation in non-adherent conditions (P2B9). Moreover, clones P5B3 and P6D4 formed more spheres than the more vimentin-positive clones P5F3 and P2B9. Therefore, EMT did not necessarily bestow enhanced sphere-forming ability. Parental OPCT-1 formed a surprisingly low number of spheres considering that this cell line comprises a mixed population of cells.

Clones P2B9 and P5F3 were less capable of forming spheres than parental OPCT-1 hence, these clones appeared to possess a smaller cancer stem/progenitor cell population than the parental cell line from which they were derived. Clones P5B3, P6D4 and P4B6 however, formed more spheres than parental OPCT-1 therefore, were likely to be more enriched with CSC/progenitor cells.

In order to investigate the expression of E-Cadherin and vimentin in the spheres formed by the OPCT-1 clones and parental OPCT-1, the spheres were stained by immunofluorescence (Figure 6.6). Vimentin expression in the spheres was not only of interest with regards to the occurrence of EMT but also because vimentin is known to be expressed by primitive (i.e. stem/progenitor) cells (Langa et al., 2000) hence in this case, vimentin expression may have also identified CSCs.
Figure 6.5: Demonstrating the results of the Sphere-forming Assay performed on clones P5B3, P6D4, P5F3, P2B9, P4B6 and parental OPCT-1 and example images of the spheres. Cells were plated at clonal density in normal media, in ultra-low adherent 24-well plates and cultured over a period of 12 days prior to enumerating the spheres. \( p < 0.0001 \), Friedman statistic < 41.57. \( n=3 \). Representative images. Image magnification at x10 and x20.
Figure 6.6 A: Demonstrating the expression of E-Cadherin and vimentin in spheres formed by clones P5B3, P6D4, P5F3, P2B9, P4B6 and parental OPCT-1. Cells were plated at clonal density in normal media, in ultra-low adherent 24-well plates and cultured over a period of 12 days prior to conducting immunofluorescence with a murine monoclonal anti-E-Cadherin and a rabbit polyclonal anti-vimentin antibody. Secondary antibody and nuclear staining were achieved as previously described in Figure 5.1. (n=2) Representative images. Image magnification at x40.
Vimentin-positive cells were observed in the spheres generated by every cell type (Figure 6.6 A). Furthermore, the distribution of the vimentin-positive cells was non-uniform as they were present in both the centre and on the surface of the spheres for all of the cell types. It should be noted that the representative images do not necessarily depict the diversity of the localisation of vimentin-positive cells for each cell type.

Surprisingly, clone P5B3 formed spheres with a relatively high number of vimentin-positive cells. This was unexpected as this clone possessed the smallest population of vimentin-positive cells in previous assays. However, the predominantly central location of the vimentin-positive cells present in the spheres of clone P5B3 may imply that rather than cells which had undergone EMT, these vimentin-positive cells may have represented primitive cells. This is supported by previous IF studies with the clones in 2D culture which demonstrated that EMT occurred primarily on the periphery of the colonies (See Chapter 5 Figure 5.1). However, further experiments would undoubtedly be required in order to support this hypothesis.

Consistent with what was observed in 2D culture, clone P4B6 formed spheres with the largest population of vimentin-positive cells. Spheres formed by clone P6D4 demonstrated the highest levels of E-Cadherin and the lowest levels of vimentin. Interestingly, E-Cadherin expression was low on the perimeter of most of the spheres.

Figure 6.6 B illustrates that minimal background staining was observed with the isotype control antibodies and that such staining was distinct from the staining observed with the test antibodies.
6.2.3.3 Self-renewal Assay

The self-renewal assay is an extension of the sphere forming assay which assesses self-renewal based on the ability of cells from disaggregated spheres to re-form spheres following re-seeding at clonal density in non-adherent conditions. Since a property of stem cells (and cancer stem cells) is their ability to self-renew infinitely, the spheres formed by stem cells and CSCs should be capable of re-forming following countless passages in vitro. This assay is believed to distinguish between spheres formed by progenitor cells and spheres formed by stem cells because progenitor cells lack self-renewal capacity, a property unique to stem cells (Pastrana et al., 2011).

This assay was conducted on the OPCT-1 clones and parental OPCT-1 in order to ascertain whether any of these cells contained a population of self-renewing cells and whether self-renewal corresponded with vimentin-positivity i.e. EMT.

Highly statistically significant differences in primary (p < 0.0001, Kruskal-Wallis statistic < 54.60) and secondary (p < 0.0001, Kruskal-Wallis statistic < 37.74) sphere-formation were observed across the clones. As can be observed, the patterns of primary and secondary sphere-formation were distinct (Figure 6.7). Surprisingly, clone P4B6, which formed the most primary spheres, did not form the largest number of secondary spheres. Furthermore, on average, clone P5B3 formed the most secondary spheres of the clones, however, was not the most capable of primary sphere-formation. Interestingly, although not the most proficient at primary sphere-formation, Parental OPCT-1 was, on average, the most capable of forming secondary spheres. The clone with the most consistent sphere-forming abilities across the assays was clone P2B9, which formed the least primary spheres and appeared incapable of secondary sphere-formation, hence, self-renewal.

The results of this assay implied that self-renewal ability did not correlate with vimentin positivity; on the contrary, with the exception of clone P4B6, secondary sphere-forming ability (self-renewal) of the clones was almost inversely proportional to vimentin-positivity. The cells most capable of self-renewal were parental OPCT-1 and clone P5B3, suggesting that clone P5B3 potentially contains the largest population of cancer stem cells. However, this hypothesis requires further confirmation.
Figure 6.7: Demonstrating the results to the Self-renewal Assay performed on clones P5B3, P6D4, P5F3, P2B9, P4B6 and parental OPCT-1 and example images of primary and secondary spheres. Cells were plated at clonal density in normal media, in ultra-low adherent 24-well plates and cultured over a period of 12 days prior to enumeration, disaggregation and re-plating at clonal density for 12 days prior to counting secondary spheres. The red cross demonstrates that clone P2B9 was incapable of secondary sphere-formation. (n=3) Representative images. Image magnification at x10. Primary sphere-formation (p < 0.0001, Kruskal-Wallis statistic < 54.60). Secondary sphere-formation (p < 0.0001, Kruskal-Wallis statistic < 37.74).
6.2.4 Evaluation of Aldehyde Dehydrogenase 1 Activity

Aldehyde dehydrogenase 1 (ALDH1) is an enzyme involved in stem cell survival and early differentiation and as such, has been exploited to identify adult tissue stem cells. Recently, ALDH1 activity has also been utilised to identify cancer stem cells and several studies have associated ALDH1<sup>hi</sup> populations with increased migration, drug resistance and tumourigenicity (Chute <i>et al.</i>, 2006; Ginestier <i>et al.</i>, 2007; Charafe-Jauffret <i>et al.</i>, 2009; Douville <i>et al.</i>, 2009; Jiang <i>et al.</i>, 2009; Tanei <i>et al.</i>, 2009). In order to investigate a possible relationship between cancer stem cells and EMT, the ALDH1 activity of the clones and parental OPCT-1 was assessed using a commercially available, flow cytometry-based assay.

The results of the Aldefluor™ assay with example flow cytometric data of the clone with the smallest ALDH1<sup>hi</sup> population, P5B3, and the clone with the largest ALDH1<sup>hi</sup> population, P6D4, are shown in Figure 6.8. The observed differences in the percentage of ALDH1<sup>hi</sup> cells among the clones were statistically significant, as determined using a non-parametric, Kruskal-Wallis ANOVA (p < 0.0244, Kruskal-Wallis statistic < 12.89) (Figure 6.8). The flow cytometric data shown, illustrate that ALDH1<sup>hi</sup> cells presented as a shift in the population rather than a distinct population of cells. This shift is typical of cells from solid malignancies and is consistent with the results observed with the recommended control cell line, SK-BR-3 (data not shown). This assay elucidated that ALDH1<sup>hi</sup> cells were present in all of the clones and parental OPCT-1 cells.

The mean percentages of ALDH1<sup>hi</sup> cells present in each cell line are shown in Table 6.2. Almost half of the cells present in clone P6D4, the second least vimentin-positive clone, expressed high levels of the enzyme aldehyde dehydrogenase 1. Clone P4B6, the most vimentin-positive clone, possessed the second highest population of ALDH1<sup>hi</sup> cells however, clone P2B9, the second most vimentin-positive clone, had the second lowest population of ALDH1<sup>hi</sup> cells. Clone P5B3, which expressed the lowest levels of vimentin, also had the smallest population of ALDH1<sup>hi</sup> cells.

Overall, this assay failed to demonstrate a direct correlation between vimentin-positivity and ALDH1 activity. Once again, these results reveal the importance of examining a range of EMT phenotypes when investigating a relationship between epithelial to mesenchymal transition and cancer stem cells.
Table 6.2 Displaying the average percentage of ALDH1$^{hi}$ cells present in parental OPCT-1 and five clones derived from this cell line.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mean % ALDH1$^{hi}$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>37.31%</td>
</tr>
<tr>
<td>P5B3</td>
<td>13.65%</td>
</tr>
<tr>
<td>P6D4</td>
<td>45.73%</td>
</tr>
<tr>
<td>P5F3</td>
<td>35.54%</td>
</tr>
<tr>
<td>P2B9</td>
<td>34.18%</td>
</tr>
<tr>
<td>P4B6</td>
<td>40.38%</td>
</tr>
</tbody>
</table>
6.2.5 Investigation of Invasive and Migratory Potential

6.2.5.1 Matrigel™ Invasion Assay

Nowadays, it is widely accepted that cancer cells induce the latent EMT programme in order to break away from the primary tumour, invade surrounding tissues and metastasise to distant sites (Acloque et al., 2009; Kalluri and Weinberg, 2009; Polyak and Weinberg, 2009; Thiery et al., 2009; Scheel and Weinberg, 2012). Therefore, EMT is largely implicated in metastasis. However, it is also accepted that cells must possess necessary self-renewal capabilities in order to colonise at distant sites. As such, cancer stem cells are also implicated in metastasis (Sarkar et al., 2009; Sapieri and Fodde, 2012; Scheel and Weinberg, 2012). In order to confirm a link between EMT and invasiveness, Matrigel™ invasion assays were conducted on the OPCT-1 clones and parental OPCT-1 and the percentage invasion was calculated for each (Figure 6.9; Table 6.3).

The OPCT-1 clones and parental OPCT-1 exhibited significant differences in invasiveness according to the Matrigel™ invasion assay, statistically analysed using a non-parametric Friedman ANOVA (p < 0.0024, Friedman statistic < 18.49).

As anticipated, the most vimentin-positive clone (P4B6) was clearly the most invasive, with a median percentage invasion of 17.5. (Figure 6.9; Table 6.3). However, the second most vimentin-positive clone (P2B9) appeared to be the least invasive clone, with a median of 4.5% invasion. Therefore, there was no direct correlation between vimentin-positivity and invasiveness.
Figure 6.9: Demonstrating the results of the Matrigel™ Invasion Assay. Cells were plated into BD Biocoat™ control or Matrigel™ inserts and incubated overnight prior to removing non-invading cells, fixing invading cells, PI staining and enumerating the invading cells using a C.T.L ELISPOT plate reader. Percentage invasion was determined by dividing the number of cells on the Matrigel™ inserts by the number on the control inserts and multiplying by 100. Data are represented as median ± quartile range. \( p < 0.0024, \text{Friedman statistic} < 18.49 \). \((n=3)\).

Table 6.3 The average percentage invasion of parental OPCT-1 and five clones derived from this cell line.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Median % Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>3.5%</td>
</tr>
<tr>
<td>P5B3</td>
<td>6%</td>
</tr>
<tr>
<td>P6D4</td>
<td>6.5%</td>
</tr>
<tr>
<td>P5F3</td>
<td>5%</td>
</tr>
<tr>
<td>P2B9</td>
<td>4.5%</td>
</tr>
<tr>
<td>P4B6</td>
<td>17.5%</td>
</tr>
</tbody>
</table>
6.2.5.2 *In vitro* Scratch Assay

The *in vitro* scratch assay is a simple method that is widely used to measure cell migration (Liang *et al.*, 2007). The method usually involves creating a “scratch” in a cell monolayer, capturing images of the closure of the scratch and quantifying the migration rate of the cells. However, in this study the nature of the cells which migrated into the scratch was of more interest than the rate at which they migrated. Therefore, this assay was modified in order to immunofluorescently stain the cells prior to complete closure of the scratch. To that end, the assay was conducted in fluorescence compatible 96 well-plates in which IF could be performed immediately after the assay (12 hours after the scratch was introduced) with antibodies directed against vimentin and E-Cadherin.

Figure 6.10 clearly demonstrates that the vimentin-positive cells in clones P2B9 and P4B6 migrated into the scratch. Furthermore, the vimentin-negative cells on the edge of the scratch of clone P4B6 were also negative for E-Cadherin. Downregulation of E-Cadherin is an early event in EMT therefore, the vimentin-negative cells in clone P4B6 may have been activating the EMT programme. This is supported by the presence of vimentin-low cells which were presumably in the process of transitioning (indicated by orange arrows). Vimentin positive cells were not observed in or along the scratches of clones P5B3, P6D4, P5F3 and parental OPCT-1 therefore, it appeared that only the EMT-derived cells in clones P2B9 and P4B6 possessed enhanced migratory capacity.
**Figure 6.10:** Demonstrating the results of the modified in vitro Scratch Assay with dual immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9, P4B6 and parental OPCT-1 stained with E-Cadherin (red) and vimentin (green), conducted in fluorescence-compatible 96 well plates as described in Figure 5.1. (n=3). Representative images. Image magnification at x20. Orange arrows demonstrate P4B6 cells with low vimentin-expression which may have been in the process of transitioning via EMT.
6.2.6 *In vivo* Assays

6.2.6.1 Evaluation of *in vivo* Tumourigenesis

Cancer stem cells are believed to be solely responsible for tumourigenesis, tumour differentiation, tumour maintenance and tumour progression (Moltzahn *et al.*, 2008). Several groups have identified, isolated and injected CSCs into immunocompromised mice in order to assess their tumourigenicity and differentiation potential compared with CSC-depleted populations (Ailles and Weissman, 2007; Clevers, 2011). In 2008, Mani *et al.*, demonstrated that the induction of an EMT in breast cancer cells generated cells with the properties of stem cells (Mani *et al.*, 2008). In order to assess the correlation between EMT and enhanced tumourigenesis in a prostate cancer model, four OPCT-1 clones; P5B3, P6D4, P2B9 and P4B6 and parental OPCT-1 were injected with Matrigel™, subcutaneously into the right flank of male, athymic nude mice and monitored over a period of 52 days. On completion of the experiment (day 52) the mice were euthanised and the tumours were excised, snap-frozen in OCT, cryostat-sectioned and stained by immunofluorescence.

Clear differences were observed in the tumourigenicities of the clones (Figure 6.11). Clone P4B6, the most vimentin-positive clone, formed tumours in every mouse which were significantly larger than those formed by parental OPCT-1 and this was the only clone to form tumours in all of the mice injected. Furthermore, the tumours formed by clone P4B6 were at least twice the size of those formed by the other cells. Clone P2B9, the second most vimentin-positive clone, however, only formed a tumour in one of five mice. Clone P5B3, the least vimentin-positive clone, did not form tumours in any of the six mice injected. However, clone P6D4, the second least vimentin-positive clone, was the second most tumourigenic of the clones and formed tumours in three out of five mice. Although only the most vimentin-positive clone (P4B6) was more tumourigenic than parental OPCT-1, taken together, these results failed to directly demonstrate a correlation between EMT and tumourigenesis as the rank order of tumourigenicity did not increase with vimentin-positivity (Figure 6.12). Once again, this study demonstrated the importance of using a range of cells to investigate an association between EMT and aggressivity as clones P5B3 and P4B6 supported the notion that EMT confers an aggressive phenotype however clones P6D4 and P2B9 contradicted this.
Figure 6.11: Demonstrating the results of the in vivo Tumourigenesis Assay with images of excised tumours. Clones P5B3, P6D4, P2B9, P4B6 and parental OPCT-1 were injected subcutaneously into the right flanks of male athymic nude mice, six mice per cell line. Tumour growth was monitored using calliper measurements and mice were euthanised once one of the tumours reached 1cm in diameter. Data are represented as the mean tumour area ± STDEV. A student T-test was used to determine statistical significance in the difference between clone P4B6 and parental OPCT-1, for the last three measurements. (n=1).

- P5B3: (0/6)
- P2B9: (1/5)
- P6D4: (3/5)
- Parental: (5/6)
- P4B6: (4/4)

Increase in tumourigenicity

Figure 6.12: Demonstrating the rank order of four OPCT-1 clones and parental OPCT-1 with regards to tumourigenicity in male athymic nude mice.
6.2.6.2 Investigating the in vivo Phenotype

In order to evaluate whether the clones had maintained the same proportions of vimentin-positive cells in vivo and examine the distribution of the vimentin-positive cells within the tumours, the tumours were sectioned and stained by immunofluorescence. However, due to the fact that vimentin is highly conserved and the antibody used also recognises murine vimentin, it was necessary to ensure that murine cells were not present in the tumours. To that end, sections were dual-stained with anti-vimentin and anti-H2Kb (an antibody which recognises murine MHC class I) (Figure 6.13) prior to staining subsequent sections with vimentin and E-Cadherin (Figures 6.14 and 6.15). Murine cells were not detected in any of the tumour-sections (Figure 6.13). Therefore, it was possible to proceed with dual-staining the tumour sections with E-Cadherin and vimentin antibodies (Figures 6.14 and 6.15).

Figure 6.14 demonstrates low-magnification images which provide an overview of the tumour sections stained with E-Cadherin and vimentin and isotype control antibodies. With regards to isotype staining, the level of background staining achieved with the isotype control antibodies was acceptable and distinct from the staining patterns achieved with the test antibodies. This experiment revealed that the cells did not all maintain their phenotypes, with regards to E-Cadherin and vimentin positivity, in vivo. This is because there were no vimentin-positive cells observed in the tumour section from clone P2B9, the population of vimentin-positive cells for clone P6D4 was far greater in vivo than in vitro and there were fewer vimentin-positive cells observed in clone P4B6 in vivo. This figure reveals that clone P6D4 appeared to form glandular structures – some of which were surrounded by a layer of vimentin-positive cells. The organised structure of these tumours was possibly indicative of a population of stem-like cells, capable of differentiating into distinct cell types. The vimentin-positive cells present in the P4B6 tumours were predominantly localised close to the periphery of the tumour, as would be expected of cells activating EMT as a means to escape and metastasise. The vimentin-positive cells in the tumours of parental OPCT-1 were also predominantly situated towards the periphery however there were also several scattered throughout.

A closer view of the stained tissue sections is provided in Figure 6.15. From this figure, it is evident that clone P6D4 formed gland-like structures with areas of high E-Cadherin positivity (red) as well as structures surrounded by vimentin-positive cells (green). Even at high magnification, no vimentin-positive cells were observed in the tumour formed by
clone P2B9. It is important to note that the “vacuoles” observed in the P2B9 tumour are likely to have been artefacts created during sectioning as this tumour was the smallest and most difficult to section. Clone P4B6 and parental OPCT-1 formed tumours with tight cell-cell contact and fewer glandular structures than clone P6D4 although structures were also observed in these tumours. Please refer to Appendix VI for isotype control images at x10, x20 and x40 magnification.
Figure 6.13: Demonstrating the immunofluorescent staining of tumour sections derived from clones P5B3, P6D4, P2B9, P4B6 and parental OPCT-1 and murine kidney as a positive control for H2Kb expression. Sections were labelled with murine monoclonal anti-H2Kb and rabbit polyclonal anti-vimentin. Secondary antibody staining was achieved using anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 antibodies. Nuclear staining was achieved as described in Figure 5.1. (n=2). Representative images, x20 magnification.
**Figure 6.14:** Demonstrating the immunofluorescent staining of tumour sections derived from clones P5B3, P6D4, P2B9, P4B6 and parental OPCT-1. Sections were stained with anti-E-Cadherin (red) and anti-vimentin (green) (left) and murine and rabbit isotype control antibodies (right). Secondary antibody and nuclear staining were achieved as described in Figure 5.1. (n=2). Representative images. x4 magnification.
Figure 6.15: Demonstrating the immunofluorescent staining of tumour sections derived from clones P5B3, P6D4, P2B9, P4B6 and parental OPCT-1. Sections were stained with anti-E-Cadherin (red) and anti-vimentin (green). Secondary antibody and nuclear staining were achieved as described in Figure 5.1. (n=2). Representative images. x10, x20 and x40 magnification.
6.2.7 Summary of Findings

The findings from this study are summarised in Table 6.4. The most vimentin positive clone (P4B6) exhibited the most aggressive phenotype overall as, with the exception of docetaxel resistance and self-renewal ability, this clone ranked highest in all assays. The second most vimentin-positive clone (P2B9) however, demonstrated the least aggressive phenotype overall. Clone P5B3, which was the least vimentin-positive, also demonstrated an overall non-aggressive phenotype. The second least vimentin-positive clone (P6D4) however, was the second most aggressive overall and demonstrated higher docetaxel resistance than clone P4B6. Clone P5F3 represented an intermediate phenotype, which was neither the least, nor the most positive in any of the assays conducted.

Table 6.4 Summarising the results to the characterisation assays conducted on the OPCT-1 clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Proliferation</th>
<th>Docetaxel Resistance</th>
<th>Colony Formation</th>
<th>Sphere Formation</th>
<th>Self-Renewal Activity</th>
<th>ALDH1 Activity</th>
<th>Matrigel™ Invasion</th>
<th>Tumourigenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5B3</td>
<td>+</td>
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Key: Negative - V.low -/+ Low + Moderate ++ High +++ V.high ++++

Overall these results demonstrate the importance of using a range of phenotypes to investigate a relationship between EMT and an aggressive CSC phenotype as, when all of the clones are considered, it is evident that there is not always a direct relationship between the two phenomena.
6.3 Discussion

Previous work led to the derivation of five OPCT-1 clones which were subsequently phenotyped according to their expression of EMT and CSC-associated markers (Chapters 4 and 5). The expression of vimentin differed significantly among these clones, therefore, as vimentin-positivity has been shown to correspond with EMT in carcinoma cells, the five OPCT-1 clones provided a model to investigate the properties of epithelial to mesenchymal transition in human prostate cancer cells. Using a range of different assays, this study aimed to thoroughly interrogate these clones in order to establish whether or not EMT of prostate cancer cells generates cells with the properties of stem cells.

In order to investigate a possible link between EMT and enhanced proliferative capacity, a property attributed to prostate cancer stem cells (Collins et al., 2005; Jeter et al., 2011), three distinct proliferation assays were conducted on the five OPCT-1 clones. Despite differences in the methods of assessing proliferation, the ViaLight® Plus assay and the Thymidine assay generated analogous results. Furthermore, these results were consistent with microscopic observation of the clones, with regards to the speed at which they reached confluency. The MTT assay however, generated results that were inconsistent with microscopic observations hence, this assay was not exploited for further experiments.

The ViaLight® Plus and Thymidine proliferation assays revealed that the most vimentin-positive clone, P4B6, was also the most proliferative. However, these assays also revealed that the second most vimentin-positive clone, P2B9, was one of the least proliferative, along with the least vimentin-positive clone, P5B3. If one were to examine the proliferation of the least vimentin positive clone, clone P5B3, and compare it with the proliferation of the most vimentin positive clone, clone P4B6, one would assume that the EMT process confers an increase in proliferation. However, the fact that the two most vimentin-positive clones, P2B9 and P4B6, demonstrated vast differences in their abilities to proliferate confirms that this is not necessarily the case. Therefore, since proliferative capacity did not increase corresponding to vimentin-positivity overall, there did not appear to be a direct relationship between EMT and enhanced proliferation of prostate cancer cells. This finding emphasises the importance of investigating a number of clones with a range of EMT phenotypes as opposed to simply comparing an EMT-negative population.
with an artificially-induced population of EMT-derived mesenchymal cells – as several studies have done (Mani et al., 2008; Kong et al., 2010; Li and Zhoul, 2011; Moustakas and Heldin, 2012).

Paradoxically, cancer stem cells are widely believed to possess enhanced proliferative capacity (although they may also undergo periods of quiescence) however, induction of EMT has been shown to be associated with reduced proliferation and a state of growth arrest (Postigo, 2003; Vega et al., 2004; Brabletz, 2012). Since clone P4B6 was the most vimentin (EMT)-positive and the most proliferative, these data corresponded with the notion that EMT confers enhanced proliferative capacity, perhaps by bestowing stem-like properties. Conversely, since P2B9, the second most vimentin-positive clone, was the least proliferative, this finding corresponded with the studies which showed that EMT confers reduced proliferation and growth arrest. Therefore, there appeared to be more than one cell type generated via EMT across the clones.

Cancer stem cells and EMT-derived mesenchymal-like cells are believed to have serious implications for cancer therapy (See Section 1.6.3.8). Through a variety of different mechanisms, including expression of ABC transporters, active DNA repair, increased expression of anti-apoptotic proteins and high ALDH1 activity, both populations have been shown to be highly resistant to chemotherapeutic agents (Conticello et al., 2004; Hollier et al., 2009; Alison et al., 2012; Tiwari et al., 2012). As such, these populations present a serious problem for cancer biologists and oncologists hence, further characterisation of CSCs and EMT-derived cells to determine viable therapeutic targets is of utmost importance. During this study, the sensitivity of the five OPCT-1 clones to the first line advanced prostate cancer chemotherapeutic agent, docetaxel, was assessed in attempt to ascertain a possible correlation between vimentin-positivity and chemotherapeutic resistance.

Using parental OPCT-1 as a model to ascertain the IC50 dose for use in further studies, the Thymidine proliferation assay was found to be most suitable for assessing the chemotherapeutic sensitivity of the OPCT-1 clones. Using docetaxel, the Thymidine assay revealed that statistically significant variation in chemotherapeutic resistance was evident among the clones, however, resistance did not correspond with vimentin-positivity. On the contrary, the most drug resistant clone, P6D4, expressed the second lowest levels of vimentin whereas the second most vimentin-positive clone, P2B9, was the second most
sensitive to docetaxel treatment. Surprisingly, the most vimentin-positive clone, P4B6, proved to be more than twice as sensitive to docetaxel treatment as the most resistant clone, P6D4. Overall, the least vimentin-positive clone, P5B3, proved to be the most sensitive to docetaxel treatment however, when taking all other results into consideration, this result was not considered to be due to low vimentin-positivity.

Previous work revealed that clone P6D4 expressed the highest levels of the transcription factor Nanog (Chapter 5). Recently, CD133+/CD44+/integrin α2β1+ putative prostate cancer stem cells, isolated from the PC3 cell line, were shown to exhibit increased expression of Nanog (Gong, et al., 2012). Furthermore, Nanog has been shown to promote cancer stem cell characteristics, such as enhanced clonal growth and tumour regenerative capacity, and drug resistance via upregulation of ALDH1 (Jeter et al., 2011). Therefore, since clone P6D4 was the most Nanog-positive and drug resistant clone, but one of the least vimentin-positive clones, it is plausible that this clone may be enriched for prostate cancer stem cells, which are distinct from the stem-like cells generated via EMT.

Interestingly, although docetaxel is an anti-mitotic chemotherapy medication (Tannok et al., 2004), therapeutic sensitivity did not correspond with high proliferative capability and vice versa, evidenced by the fact that the most proliferative clone was not the most sensitive to docetaxel treatment (P4B6) and the second most sensitive clone was the least proliferative (P2B9).

Since therapeutic resistance was not shown to correlate with vimentin-positivity, these results suggested that EMT of prostate cancer cells did not greatly enhance therapeutic resistance. Hence, this study did not provide evidence to support the consensus that EMT confers acquired drug resistance (Hollier et al., 2009; Tiwari et al., 2012). However, since clone P4B6 and P2B9 comprise a mixed population of epithelial cells, cells undergoing EMT and EMT-derived-mesenchymal cells, the drug sensitivity may have only been attributed to the epithelial fraction, which makes up the majority of the clones. As such, in order to accurately ascertain a link between EMT and drug sensitivity, perhaps it would be necessary to conduct drug assays on a pure population of cells which have undergone EMT. That said however, if EMT were to confer drug resistance, one would expect a clone with such a high proportion of EMT-derived cells (P4B6) to demonstrate higher resistance than a clone with a low number of these cells, derived from the same cell line (P6D4). However, this was not the case. In order to address the chemotherapeutic
sensitivity of a pure EMT-derived population, the drug sensitivity of the 100% vimentin-positive/E-Cadherin negative OPCT-1 clone (discussed in Chapter 5) will be assessed in comparison to the five OPCT-1 clones with mixed vimentin/E-Cadherin expression, in future studies.

Akin to normal stem cells, cancer stem cells have been shown to possess enhanced clonogenic capacity (colony forming ability). Therefore, in order to address a possible correlation between EMT and stemness, the clonogenicity of the five OPCT-1 clones was investigated. According to this assay, the OPCT-1 clones differed very significantly with regards to clonogenicity. Interestingly, clonogenicity corresponded with vimentin-positivity hence, EMT, as the two most vimentin-positive clones, P4B6 and P2B9, were also the most clonogenic and the least vimentin-positive clone, P5B3, was the least clonogenic. However, the colonies formed by the two most vimentin-positive/clonogenic clones differed notably in that clone P4B6 formed the largest colonies of all the clones whereas clone P2B9 formed the smallest colonies. The results obtained in this study were consistent with another study on prostate cancer cell lines which also demonstrated that cells with an EMT phenotype possessed enhanced clonogenic capacity (Kong et al., 2010).

Based on the reported capacity to evaluate self-renewal and differentiation at the single-cell level in vitro, the sphere-forming assay is one of the most widely used assays for the identification and enrichment stem cells and cancer stem cells (Dontu et al., 2003; Mani et al., 2008). Therefore, the stemness of the five OPCT-1 clones was assessed with regards to their sphere-forming potential. This assay revealed that all five clones were capable of sphere-formation in non-adherent conditions and highly significant differences in the ability of the clones to form spheres were observed. However, although the most vimentin-positive clone, P4B6, was by far the most capable of anchorage independent growth (sphere-formation), the second most vimentin-positive clone was by far the least capable hence, vimentin-positivity did not correlate with sphere-forming ability. Therefore, EMT did not appear to bestow enhanced sphere-forming abilities upon these prostate cancer cells. As with the proliferation assays, this experiment highlighted vast behavioural differences between the two most vimentin-positive clones, thereby indicating that EMT may give rise to distinct cell types in different settings. Interestingly, clone P6D4, formed the second highest number of spheres across the three assay repeats. This finding provided additional evidence that the P6D4 clone was enriched for cancer stem cells.
Overall, the data obtained from the sphere-forming assay conflicted with other studies which demonstrated enhanced sphere-formation by cells with an EMT phenotype (Mani et al., 2008; Kong et al., 2010).

Following the sphere-forming assays, the spheres were immunofluorescently stained with E-Cadherin and vimentin antibodies to assess the distribution of these proteins among the spheres and ascertain whether or not the spheres were enriched for vimentin-positive cells. As previously stated, vimentin is also expressed by primitive cell types prior to differentiation (Langa et al., 2000) therefore, in addition to EMT-derived cells, it is possible that vimentin may also identify cancer stem cells. Immunofluorescence revealed that all of the clones formed spheres containing vimentin-positive cells, with clone P4B6 containing the most and clone P6D4 containing the least. Interestingly, with the exception of the spheres formed by clones P6D4 and P2B9, (which approximately maintained the same proportion of vimentin-positive cells) compared to 2D culture, all spheres were enriched for vimentin-positive cells. Unexpectedly, the majority of the spheres showed low expression of E-Cadherin towards the periphery. However, since E-Cadherin loss is a hallmark of EMT (Zeisberg and Neilson, 2009), perhaps the downregulation of this protein on the periphery of the spheres was indicative of EMT in some cases.

Of particular interest was the fact that the least vimentin-positive clone in 2D culture (P5B3) formed spheres with multiple vimentin-positive clones, located towards the centre of the spheres. Since EMT is believed to occur predominantly at the periphery of tumours (Thiery et al., 2009), perhaps the cells located towards the centre of the spheres represented a cancer stem cell component in clone P5B3. However, thus far, all assays failed to reveal that clone P5B3 exhibited a cancer-stem cell signature, therefore, this hypothesis undoubtedly requires further evidence. Investigating the expression of other EMT-associated markers such as fibronectin and N-Cadherin may confirm that the vimentin-positive cells in clone P5B3 spheres were not EMT-derived therefore may provide further evidence to suggest that they represented a CSC population. However, since clone P6D4, which previously demonstrated cancer stem cell attributes, contained a low population of vimentin-positive cells within the spheres, perhaps CSCs do not necessarily express this intermediate filament. Therefore, it would be interesting to ascertain whether putative prostate cancer stem cells, with the cell surface phenotype identified by Collins et al., 2005 (CD133+/CD44+/integrin α2β1hi), express vimentin.
Self-renewal ability is believed to be a property unique to stem cells and cancer stem cells (Zhao et al., 2008). As such, the ability of the OPCT-1 clones to self-renew following sphere-culture was investigated in an attempt to explore a correlation between stemness and EMT. Surprisingly, the least vimentin-positive clone, P5B3, formed the most secondary spheres, thus, was the most capable of self-renewing and the second most vimentin-positive clone, clone P2B9, was incapable of forming secondary spheres in two out of three assays. Therefore, vimentin-positivity certainly did not correspond with self-renewal abilities and, with the exception of clone P4B6 which formed the second largest number of secondary spheres, self-renewal was almost inversely proportional to vimentin-positivity.

Aldehyde dehydrogenase (ALDH1) activity has been shown to be increased in stem cells and cancer stem cells hence, this enzyme provides a marker for cancer stem cells from several malignancies (Chute et al., 2006; Ginestier et al., 2007; Charafe-Jauffret et al., 2009; Douville et al., 2009; Jiang et al., 2009; Tanei et al., 2009; Jeter et al., 2011). This study revealed that all five of the OPCT-1 clones contained a population of ALDH1hi cells, the percentage of which differed significantly among the clones. Although the most vimentin-negative clone also expressed the lowest levels of ALDH1, there was no direct correlation between ALDH1 activity and vimentin-positivity. In keeping with the previous assays, which revealed that clones P6D4 and P4B6 presented with stem-like properties, these clones were shown to express the highest ALDH1 activities. Interestingly, the levels of this enzyme corresponded with drug resistance, in that the clone with the lowest ALDH1 activity, P5B3, was also the most sensitive to docetaxel treatment and the clone with the highest ALDH1 activity, P6D4, was the most resistant to docetaxel treatment. This finding is in keeping with the fact that ALDH1 is known to play a role in drug detoxification (Dockham et al., 1997) hence, high levels would be expected to confer drug resistance. The validity of this assay in assessing stemness however, is questionable. This is because it was expressed by more than 10% of the population in every clone and up to almost 50% of the cells in clone P6D4 but CSCs are generally reported to represent a small population. Furthermore, the high percentage of ALDH1hi cells in the P2B9 clone was not in keeping with the fact that this clone had not demonstrated an overall stem-like signature according to other assays. It is worth noting that the doubts regarding the validity of ALDH1 as a bona fide CSC marker are shared with other groups, which have demonstrated that ALDH1 activity failed to select for aggressive cells in malignant...
melanoma (Prasmickaite et al., 2010) and failed to identify cancer stem cells in prostate cancer (Yu et al., 2011).

Both cancer stem cells and EMT have been implicated in invasion and metastasis and several studies have utilised Matrigel™ invasion assays to assess the invasive capacity of cell populations in vitro. The expression of matrix-degrading MMPs and vimentin by EMT-derived cells, are believed to confer invasive and migratory capacity, respectively (Kalluri and Weinberg, 2009) and cancer stem cells have also been shown to present with a mesenchymal phenotype which may assist with migration (Mani et al., 2008). In order to investigate a relationship between EMT and invasion, the five OPCT-1 clones were assessed using the Matrigel™ invasion assay. This assay revealed that the most vimentin-positive clone, P4B6, was by far the most invasive. However, clone P2B9, the second most vimentin-positive, was the least invasive. Therefore, these clones failed to demonstrate a correlation between vimentin-positivity and invasiveness.

In order to ascertain which cells were most capable of migrating into an artificial “scratch” in a confluent cell monolayer, the in vitro scratch assay (Liang et al., 2007) was modified so that it could be executed in fluorescence-compatible 96-well plates, in which IF could be conducted following completion of the assay. This study convincingly revealed that the vimentin-positive cells in clones P4B6 and P2B9 were capable of migrating into the “scratch”. Therefore, in keeping with the literature (Ivaska et al., 2007), the vimentin-expressing cells were shown to possess enhanced migratory capacity. This assay could not identify whether the vimentin-positive cells migrated into the “scratch” from a distance or whether the cells on the edge of the “scratch” underwent EMT and migrated into the scratch. However, evidence of the latter was provided as the cells on the edge of the P4B6 “scratches” did not demonstrate E-Cadherin expression and in addition, low-levels of vimentin expression were also detected in cells along the edge of these scratches.

Cancer stem cells are largely implicated in tumourigenesis and have been shown to initiate tumours in a murine model with as few as 100 cells (Al-Hajj et al., 2003). Furthermore, depletion of putative cancer stem cells from the bulk of the tumour often leads to a decrease in in vivo tumourigenicity (Al-Hajj et al., 2003). Therefore, since EMT has been shown to generate cells with the properties of stem cells (Mani et al., 2008) tumourigenesis may be expected to increase with vimentin-positivity. In order to address this hypothesis, four of the OPCT-1 clones were injected into male athymic nude mice to assess their
tumourigenic capacity in vivo. This assay revealed that the most vimentin-positive clone, P4B6, was the most tumourigenic and that the least vimentin-positive clone, P5B3, was the least tumourigenic. However, as the second most vimentin-positive clone, P2B9 was the second least capable of forming tumours in mice, tumourigenesis was not shown to correlate with vimentin-positivity. Consistent with the other assays, which revealed that clone P6D4 possessed properties attributed to CSCs, this (vimentin-low) clone was the second most tumourigenic. However, only clone P4B6 was shown to be more tumourigenic than parental OPCT-1 and the tumours that formed from this clone were significantly larger than those formed by the parental cell line from which they were derived. Therefore, as demonstrated in several of the other assays, the behaviour of clones P4B6 and P2B9 varied markedly, despite expressing the highest numbers of vimentin-positive cells.

Following completion of the tumourigenesis assay, the tumours were excised, cryostat-sectioned and immunofluorescently stained for the expression of E-Cadherin and vimentin. This assay revealed that the clones did not maintain the same proportions of vimentin-positive cells in vivo and that the distribution of vimentin-positive cells varied according to the clone. Interestingly, clone P2B9, which expressed the second highest levels of vimentin in vitro, failed to express this protein in vivo. Also, clone P4B6, in which approximately 30% of the cells were shown to express vimentin in 2D culture in vitro (Chapter 5), demonstrated lower vimentin-positivity in vivo. Therefore, the injected vimentin-positive cells either died in vivo or differentiated via MET into epithelial cells. On the contrary, clone P6D4 formed tumours with a higher abundance of vimentin-positive cells than had been observed in vitro.

In keeping with the literature, which generally reports that cells at the tumour-host interface (i.e. the periphery) activate EMT in order to break-away from the primary tumour and metastasise (Kalluri and Weinberg, 2009), the vimentin-positive cells in the P4B6 and parental tumours were predominantly located towards the periphery of the tumours. Intriguingly, clone P6D4 formed tumours with E-Cadherin-high gland-like structures and structures which were surrounded by vimentin-positive cells. Therefore, the organised nature of the tumours formed by this clone implied that there may have been a stem-cell component present. This finding was in keeping with other assays, which revealed that this clone demonstrated cancer stem cell attributes.
Throughout the duration of this study, the two most vimentin-positive clones often generated contrasting data, with clone P4B6, the most vimentin-positive, demonstrating a highly aggressive phenotype overall and clone P2B9, the second most vimentin-positive, predominantly showing a non-aggressive phenotype. Type 1 and 3 EMTs generate mesenchymal cells whereas, Type 2 EMT gives rise to fibroblasts (See Section 1.6.3) (Kalluri and Weinberg, 2009). Therefore, it is plausible that there may also be distinct EMT events that give rise to different cell products in cancer. As such, in some cases EMT may confer stem-like properties, whereas in others, it may generate non-stem-like progeny such as fibroblasts.

In 2010, Battula and colleagues revealed that EMT-derived cells exhibit multi-lineage differentiation potential and a gene signature similar to mesenchymal stem cells (Battula et al., 2010). Therefore, EMT of cancer cells may also generate mesenchymal stem-like cells. As such, the stem-like attributes of clone P4B6 may be bestowed by a population of mesenchymal stem-like cells. This hypothesis requires verification by flow cytometry using a panel of mesenchymal stem cell markers, which are currently under optimisation, and by demonstrating multilineage differentiation into the progeny of mesenchymal stem cells; osteoblasts, chondrocytes and adipocytes.

In summary, although the most vimentin-positive clone, P4B6, demonstrated several attributes of cancer stem cells and the most aggressive phenotype overall, conflicting data generated from the second most vimentin-positive clone, P2B9, failed to support the hypothesis that EMT generates cells with stem-like properties. Furthermore, one of the least vimentin-positive clones, P6D4, demonstrated more stem-like properties than clone P2B9. As such, this study failed to establish a definitive link between EMT and stemness in every case. However, it is evident that in some settings, EMT of prostate cancer cells does generate cells with the properties of stem cells, i.e. clone P4B6, whereas in others, such as clone P2B9, it does not. Therefore, future studies will aim to identify the cell types generated via EMT in different OPCT-1 clones and primary cultures of prostate cancer cells, in order to ascertain whether EMT of prostate cancer cells is capable of generating more than cell type and determine how these cells may be targeted, taking into consideration the need to circumvent any inherent resistance mechanisms. In addition to revealing potential evidence of distinct EMT events in human prostate cancer, this study demonstrated CSC-like attributes from a vimentin-low clone, thereby providing evidence that multiple populations of cancer stem-like cells may exist within a given tumour.
Therefore, in order to achieve permanent tumour eradication, it is essential to identify and target the underlying mechanisms governing the aggressive cancer stem cell phenotype.
Chapter 7:

Preliminary Studies on Human Prostate Cancer Patient Tissue

7.1 Introduction

Human prostate carcinoma cells are notoriously difficult to culture in vitro. Consequently, very few prostate cancer cell lines are available for research and most of these are derived from metastases. The most commonly used cell culture models of prostate cancer are the spontaneously established, metastasis-derived, cell lines PC3, DU145 and LN-CaP (Bosland et al., 1996). However, these do not represent the whole range of prostate cancer phenotypes and also fail to represent primary adenocarcinomas (Peehl, 2005). Since prostate carcinoma cells may have a finite lifespan, senescing after approximately 30 population doublings (Miki and Rhim, 2008), artificial immortalisation with viral oncogenes has also been employed to derive immortal cell lines, an example of which is the OPCT-1 cell line. However, despite the use of viral constructs, the proportion of patient samples that give rise to immortalised cultures remains disappointingly low (Miki and Rhim, 2008). Furthermore, artificial immortalisation and long-term culture are believed to alter the biological properties of cell lines hence, their usefulness as a model of prostate cancer is often debated (Peehl, 2005). As such, primary cells are widely regarded as superior culture models of prostate cancer (Peehl, 2005; Oldridge et al., 2011).

Despite compelling evidence for the role of EMT in human cancer progression, this hypothesis is not accepted by all (Garber, 2008). Controversy regarding the role of EMT in carcinogenesis has been largely due to a lack of sufficient evidence in patient tumour samples (Tarin, 2005).

Therefore, this work endeavoured to investigate the occurrence of EMT in primary cultures of high-grade human prostate carcinoma cells and sectioned prostate cancer tissue in attempt to provide further evidence of EMT in human prostate cancer.
7.2 Results

Previous studies demonstrated the occurrence of epithelial to mesenchymal transition in the transformed prostate cancer line OPCT-1 (Chapters 4-6). In order to confirm that this phenomenon had not merely occurred as a result of artificial immortalisation (with HPV genes E6 and E7) and long term cell culture of the OPCT-1 cell line, it was necessary to demonstrate evidence of EMT in non-immortalised cultured primary prostate cancer cells and sectioned high-grade prostate cancer and benign hyperplastic prostate (BPH) tissues obtained from Trans Urethral Resectioning of the Prostate (TURP) procedures.

7.2.1 Examination of Primary Stromal and Epithelial Cultures

In order to investigate EMT in primary cultures of prostate cells, fresh patient material, either high-grade PCa tissue (Gleason 8-10) or BPH tissue, was collected from TURP procedures and processed immediately following the method optimised by Professor Norman Maitland’s research group at The University of York (Collins et al., 2005), but without integrin α2β1 selection and addition of feeder cells to the epithelial fraction. Figure 7.1 summarises the work flow for the separation, culture and examination of cultured primary prostate stromal and epithelial cells.

*Figure 7.1:* Illustrating the procedures followed for the separation and investigation of epithelial and stromal populations from fresh prostate cancer and benign hyperplastic prostate samples. Tissues were collected, cut into small pieces and digested overnight in collagenase solution. The stromal cells were separated from the epithelial cells in a series of centrifugation steps and cultured in simple media with FCS. The epithelial fraction was then treated with trypsin and versine prior to culturing on collagen-coated flasks in defined serum-free media. Once confluent, cells were plated and examined by IF.
Microscopic Examination of Primary Stromal and Epithelial Cultures

Primary cultures from two benign and 13 malignant prostate tissues were derived as previously described (Figure 7.1). After approximately 1-2 weeks in culture, microscopic examination of the cultured epithelial and stromal fractions revealed distinct morphological differences between the two populations and confirmed that they had been successfully separated (Figures 7.2 to 7.5). Occasionally, a few stromal cells were observed in some of the benign epithelial cultures (indicated by red arrows in Figure 7.4) however, the serum-free conditions and cholera toxin, which provided an inhospitable environment for primary stromal cells, ensured that these cells were infrequent and did not outgrow the epithelia.

Despite the omission of irradiated murine fibroblast feeder cells, the epithelial cells isolated from malignant tissue grew well, albeit slowly, in culture (Figure 7.2). They formed predominantly dense colonies which exhibited the cobblestone morphology characteristic of epithelial cells. As demonstrated in Figure 7.2, slight variation in the cell size and density of the colonies was observed however, the cells were certainly morphologically distinct from the stromal cells isolated from malignant tissue (Figure 7.3). As anticipated, the epithelial cells isolated from BPH samples (Figure 7.4) did not grow as well as those derived from high-grade PCa tissue. They grew in looser colonies with a stressed appearance as indicated by the presence of intracytoplasmic vacuolation (indicated by black arrows) (Henics and Wheatley, 1999). Furthermore, cultures of BPH-derived epithelial cells often senesced prior to reaching confluence. In fact, both benign and malignant epithelial populations were difficult to culture as they were strongly adherent, hence resisted trypsinisation, many clumped and died during passaging and even the prostate cancer derived epithelial cells senesced after 3-4 passages. However, senescence is not atypical of primary prostate epithelial cultures as normal human prostate epithelial cells and even primary prostate cancer cells have a finite lifespan and typically undergo 30 population doublings prior to becoming senescent (Peehl, 2005; Mikki and Rhim, 2008).

As evidenced in Figure 7.3, the stromal population isolated from high-grade prostate cancer tissues consisted of multiple cell types which may have included pericytes, fibroblasts, myofibroblasts and smooth muscle cells. Contrary to the epithelial cells, these cells appeared to proliferate extremely rapidly in culture and were cultured beyond six passages without immortalisation. This was in contrast to the stromal fraction isolated from BPH tissues which also contained a mixture of cell types (Figure 7.5) but appeared to proliferate at a slower rate in culture and senesced after approximately 3-4 passages.
Figure 7.2: Representative images of cultured epithelial cells isolated from high-grade prostate cancer tissues and cultured on collagen-coated flasks in complex, defined serum-free media optimised for the promotion of self-renewal and inhibition of differentiation of primary epithelial cells. Image magnification at x10. A-H represent individual cultures of epithelial cells derived from prostate cancer patients.
Figure 7.3: Representative images of cultured stromal cells isolated from high-grade prostate cancer tissues and cultured on polystyrene flasks in simple media with 10% serum. Image magnification at x10. A-H represent individual cultures of stromal cells derived from prostate cancer patients.
**Figure 7.4:** Representative images of cultured epithelial cells isolated from BPH tissues and cultured on collagen-coated flasks in complex, defined serum-free media optimised for the promotion of self-renewal and inhibition of differentiation of primary epithelial cells. Image magnification at x10. A-D represent individual epithelial cultures derived from BPH patients. Black arrows indicate cytoplasmic vacuolation. Red arrows indicate stromal contamination.

**Figure 7.5:** Representative images of cultured stromal cells isolated from BPH tissues and cultured on polystyrene flasks in simple media with 10% serum. Image magnification at x10. A-D represent individual stromal cultures derived from BPH patients.
7.2.1.2 Examination of Primary Stromal and Epithelial Cells by Immunofluorescence

The aforementioned problems encountered during the culture of primary epithelial cells limited the investigation of these populations as it was not possible to conduct long-term studies and assays such as sphere formation, proliferation, migration and drug resistance. Therefore, the epithelial cells were only preliminarily examined by immunofluorescence.

In order to confirm that the epithelial and stromal cells had been successfully separated, dual immunofluorescence was conducted on the two populations using a pan cytokeratin antibody to identify cells of epithelial origin and a vimentin antibody to identify cells of mesenchymal origin (example images shown in Figure 7.6). The stromal cells were free from epithelial contamination, as evidenced by the lack of cytokeratin staining. However, the results for the epithelial fraction were complicated by two factors A) it was not possible to determine whether vimentin-positive cells with fibroblastoid morphology present in epithelial cultures were contaminants or if they had arisen as a result of EMT and B) the media used to maintain the epithelial cultures was optimised for the maintenance of primitive cells hence, enriched for stem and transit-amplifying (progenitor) cells which may also express vimentin (Korschning et al., 2005). Therefore, using these two markers it was not possible to distinguish between contaminating stromal cells, cells which had undergone EMT and putative prostate cancer stem/progenitor cells. As such, it is important to stress that the data provided in this chapter are preliminary and therefore, open to interpretation. It is however, apparent that the majority of vimentin-positive cells in the epithelial cultures were morphologically distinct from the cells in the stromal cultures, which were all single vimentin-positive and exhibited spindle-like morphology. In addition, several vimentin-low cells were observed in the epithelial cultures (indicated by white arrows) and these may have been in the process of upregulating vimentin, hence, transitioning via EMT.

In an attempt to address the identity of the vimentin-positive cells in the epithelial and stromal cultures, dual staining with vimentin and fibronectin antibodies was conducted on cultures from different donors (Figure 7.7). This figure demonstrates that there was more than one type of vimentin-expressing cell present in the epithelial cultures, those which were large and co-expressed fibronectin (indicated by red arrows), and those which were small and did not express this extracellular matrix protein (indicated by yellow arrows). Although it is not possible to ascertain whether the dual vimentin/fibronectin-positive cells
arose as a result of stromal contamination or EMT of the epithelial cells, it is evident that
the vimentin-positive/fibronectin-negative cells were smaller and morphologically distinct
from the dual positive cells hence, may have represented the transit amplifying/stem cell
component as opposed to mesenchymal cells. Immunofluorescent screening of stromal
populations demonstrated that all cells co-expressed vimentin and fibronectin.

As differentiated epithelial cells replace vimentin with cytokeratin upon differentiation, the
presence of vimentin in cancerous epithelial cells could denote primitive cancer cells (i.e.
cancer stem cells) or cells which are transitioning from an epithelial to a mesenchymal
state (EMT). The demonstration of vimentin/cytokeratin co-expression by cultured
prostate cancer epithelia was not sufficient proof of EMT. Nonetheless, the frequent
observation of multinucleate, vimentin-positive cells in two distinct primary cultures of
prostate cancer-derived epithelia provided exciting evidence of either EMT or putative
cancer(ous) stem/progenitor cells which merited further investigation (Figure 7.8,
multinucleate cells identified by arrows).
**Figure 7.6:** Dual staining of cultured epithelial and stromal cells isolated from high grade prostate cancer tissues stained with a pan cytokeratin antibody to identify epithelial cells and a vimentin antibody to identify stromal cells/primitive epithelial cells. Image magnification at x20. Representative images. White arrows indicate cells with low vimentin expression which may be in the process of upregulating this protein via activation of the EMT programme.

**Figure 7.7:** Dual staining of cultured epithelial and stromal cells isolated from high grade prostate cancer tissues stained with a fibronectin and vimentin antibodies to identify stromal cells. Image magnification at x20. Representative images. Red arrows indicate large dual vimentin/fibronectin-positive cells and yellow arrows indicate small, single vimentin-positive cells thus, revealing that distinct vimentin-positive populations were present in the epithelial fraction.
Figure 7.8: Demonstrating evidence of putative cancer stem/progenitor cells or EMT-derived cells in primary cultures of epithelia from two patients. Cells were stained with A) E-Cadherin/vimentin B) and C) Pan cytokeratin/Vimentin D) CD44/vimentin. Image magnification at x20. Representative images. Arrows indicate multinucleate cells co-expressing vimentin and epithelial markers (A, B, C) and vimentin and the prostate basal/intermediate/stem cell marker CD44.
7.2.1.3 Karyotyping of Stromal Cells

The observation that cultured stromal cells derived from prostate cancer specimens grew faster than those derived from BPH tissues and did not senesce after more than six passages, led to the hypothesis that the stromal fractions isolated from high grade prostate cancer tissue may have contained a population of malignant EMT-derived cells which may have been tumourigenic. Due to ethical constraints, this could not be tested by investigation of tumourigenesis in vivo. Therefore, in order to address this hypothesis and ascertain whether cancer cells were present in the stromal cultures, stromal cells isolated from one high-grade PCa tissue and one BPH tissue, as a control, were sent for preliminary karyotyping by Dr Karen Sisley at The University of Sheffield.

The karyotyping revealed that some random numerical changes and tetraploidy were observed in both samples, however, no identifiable structural alterations or specific abnormalities were noted (data not shown). Therefore, the stromal cells isolated from benign and malignant TURP tissue appeared normal (i.e. non-cancerous) overall but both had developed random alterations associated with a loss of cell cycle regulation. However, without repeating the analysis and examining a larger number of cells, it was not possible to completely rule out the changes observed. Furthermore, as the sample size was very small, these results were only preliminary hence, could not provide conclusive evidence for or against the hypothesis. Therefore, it would be necessary to expand this study and examine the karyotypes of several benign and malignant stromal populations to better address the hypothesis presented. Furthermore, amendments to the ethics regarding the use of the patient material would enable in vivo studies with the stromal cells which may provide additional evidence of in vivo tumour development to address the notion that EMT-derived cancer cells are present in stromal populations of patients with high grade prostate cancer.

In addition to karyotyping the stromal cultures, the epithelial cultures should also be karyotyped in order to confirm their benign or malignant cell origins.
7.2.1.4 Investigating Evidence of EMT in High Grade Prostate Cancer and BPH Tissue Sections by Immunofluorescence

In order to investigate evidence of EMT in an in vivo setting, frozen sections of PCa and BPH tissues were dual-stained with antibodies directed against E-Cadherin and vimentin. Although vimentin expression identifies stromal cells, co-expression of vimentin and E-Cadherin could be restricted to either primitive epithelial cells or epithelial cells undergoing EMT. Stem and transit-amplifying cells are known to represent a small population of cells in both healthy and malignant tissue (Richardson et al., 2004; Collins et al., 2005; Charruyer et al., 2009). Therefore, the observation of multiple dual-positive cells in prostate cancer tissue sections was likely to be indicative of EMT.

This study provided potential evidence of EMT in prostate cancer tissue, as evidenced by the presence of multiple dual vimentin/E-Cadherin-positive cells in malignant patient tissue sections (Figures 7.9 and 7.10 Malignant 1-5). As anticipated, the benign sample consisted of predominantly single-positive cells, with a clear distinction between epithelial and mesenchymal populations (Figures 7.9 and 7.10 Benign). On the contrary, separation of the colours (Figure 7.10) clearly illustrated that in patients 4 and 5, all of the E-Cadherin-positive (epithelial) cells co-expressed vimentin, perhaps suggesting that the epithelial cells in these samples were undergoing EMT.

Interestingly, despite the highly disorganised appearance and lack of distinct glandular structures (characteristic of high-grade prostate cancer) present in malignant patient 1, this sample appeared to present with few dual-positive cells. However, evidence of EMT was observed for some of the cells in this sample, as indicated by yellow staining, which denotes dual-positivity, predominantly observed on the edges of the colonies of E-Cadherin-positive cells (highlighted by black arrows in Figure 7.9 and circles in Figure 7.10).

Downregulation of E-Cadherin is one of the key steps involved in the EMT programme (Zeisberg and Neilson, 2009). Therefore, the observation of cytoplasmic, as opposed to cell-surface, E-Cadherin staining in the dual-positive cells present in sections from malignant patients 2 and 3, supported the notion that these cells were undergoing EMT (indicated by circles in Figures 7.9 and 7.10).
Figure 7.9: Dual immunofluorescent staining of frozen tissue sections from five high-grade prostate cancer patients and one BPH patient stained with E-Cadherin (red) and vimentin (green) antibodies. Image magnification at x10, x20 and x40. Representative images. Benign and Malignant-1-5 represent separate patients. Circles highlight regions of dual vimentin/E-Cadherin-positive cells.
Figure 7.10: Dual immunofluorescent staining of frozen tissue sections from five high-grade prostate cancer patients and one BPH patient stained with E-Cadherin (red) and vimentin (green) antibodies with separation of colours to assist identification of dual or single-positive cells. Image magnification at x20. Representative images. Benign and Malignant-1-5 represent separate patients. Circles highlight regions of low/cytoplasmic E-Cadherin expression by vimentin-positive cells.
7.2.2 Parallel Observations between Primary Cultures & OPCT-1 Cells

Throughout the duration of the study, similarities between the primary cultures and the clonally derived OPCT-1 populations were frequently observed.

Mixed primary cultures of epithelial and stromal cells appeared to grow with similar patterns to the vimentin-high OPCT-1 clone, P4B6 (Figure 7.11). Although not identical, the similarities observed in the organisation of the cell types, with the stromal/mesenchymal populations (yellow arrows) surrounding the epithelial cells (red arrows), were apparent (cell types are distinguished based on morphological differences).

Furthermore, cultured stromal cells, isolated from high grade prostate cancer tissues, frequently formed the circular patterns observed in Figure 7.12 which was mirrored by the mesenchymal cells that circled the epithelial cells in clone P4B6. This observation was also confirmed by IF, where vimentin-positive mesenchymal cells circled the cytokeratin/E-Cadherin-positive epithelial cells (Figure 7.13 A and B). As can be observed in Figure 7.13 C and D, non-fibroblastoid cells co-expressing epithelial and mesenchymal markers were observed in both primary prostate cancer epithelial cultures and clonally derived OPCT-1 cells, thus inferring that these populations may have represented cells undergoing EMT. This is further evidenced by the increased expression of vimentin by cells on the edge of the colonies in E and F which appear more fibroblastoid than the vimentin-positive cells in the centre of the colonies and are reminiscent of metastatic disease whereby cancer cells are believed to activate EMT in order to break away from the primary tumour and migrate to distant sites. In addition to these parallel observations, large cytokeratin-positive cells with nuclear vimentin expression were observed in both the primary cultures and the OPCT-1 clones (G and H).
Figure 7.11: Bright field images of cultured primary prostate cancer-derived cells and OPCT-1 clone P4B6 demonstrating similarities in the organisation of the stromal/mesenchymal cells (indicated by yellow arrows) which surrounded the epithelial cells (indicated by red arrows). Image magnification at x10. Representative images.

Figure 7.12: Bright field images of cultured primary prostate cancer-derived stromal cells and clone P4B6 demonstrating similarities in the organisation of the stromal/mesenchymal cells. Image magnification at x10. Representative images. Circled regions demonstrate the circular arrangement patterns of mesenchymal/stromal cells which frequently formed in the primary stromal cultures, despite the absence of epithelia, and perhaps reflected what was observed in clone P4B6 where the mesenchymal cells frequently surrounded the circular colonies of epithelial cells.
Figure 7.13: Demonstrating parallel observations made between primary prostate cancer cells and OPCT-1 clones by dual immunofluorescent staining with E-Cadherin/cytokeratin and vimentin antibodies. Image magnification at x20. Representative images. A, C, E and G provide evidence of EMT in primary prostate cancer epithelial cultures. B, D, F and G show parallel observations made in the P4B6 cell line which was previously shown to undergo EMT.
The OPCT-1 clones were derived and examined as a model to investigate EMT. As parallel observations were made between these clones and the primary cultures, it could be argued, and indeed suggested, that EMT was observed in primary cultures of prostate cancer epithelia. However, it is important to acknowledge that these results are preliminary, on a limited sample set, and that further work on clonally derived populations would be required in order to conclusively provide evidence of EMT in primary cultures of prostate cancer cells.

7.3 Discussion

Epithelial to mesenchymal transition is a complex biological process which is reported to be critical for the acquisition of an invasive and metastatic phenotype by carcinoma cells. Previous work provided evidence of this phenomenon in the human prostate cancer cell line OPCT-1 (Chapter 4). In addition, model clones, representing a range of phenotypes with respect to EMT, were derived and thoroughly interrogated in order to investigate a possible correlation between EMT and the acquisition of stem-like traits (Chapters 4, 5 and 6).

Since artificial immortalisation, employed to generate the OPCT-1 cell line, and long-term in vitro cell culture are known to impact on the biological properties of cancer cells, the usefulness of cell lines for the investigation of cancer stem cells has been questioned hence, primary cultures are believed to be superior as they are thought to better reflect the characteristics of the original tissues (Peehl, 2005; Miki and Rhim, 2008).

Earlier work attempted to identify and isolate putative prostate cancer stem cells with the marker profile CD133+/CD44+/integrin α2β1hi (determined by Collins et al., 2005) from primary prostate cancer cell cultures, however, this was to no avail (Chapter 3). Consequently, the focus of this study shifted to investigating epithelial to mesenchymal transition in human prostate cancer. Having established methods to identify EMT-derived cells in prostate cancer cell lines, the primary cultures were re-visited in a preliminary attempt to reveal evidence of EMT in primary prostate cancer cells. In addition, tissue sections derived from benign and malignant patient samples were examined for potential evidence of EMT. Intriguingly, interrogation of primary cultures revealed similarities between these populations and the EMT-positive OPCT-1 clone P4B6.
Prior to investigating EMT in primary cells, it was necessary to establish individual cultures of epithelial and stromal cells and confirm that these were free from contamination with the undesired cell types. Epithelial cells are known to express cytokeratin intermediate filaments, hence, demonstration of cytokeratins in primary cultures is positive confirmation of epithelial origin and is easily accomplished using immunochemical methods such as immunohistochemistry and immunofluorescence (Peehl, 2002). Conversely, mesenchymal cells and fibroblasts are known to express vimentin intermediate filaments hence, vimentin-positivity may be used to identify stromal populations, which are predominantly comprised of fibroblasts and smooth-muscle cells (Peehl and Sellers, 1997). However, whilst mesenchymal cells do not express cytokeratins, identification of pure epithelial cultures is complicated by the fact that epithelial cells from different tissues of both benign and malignant origin have frequently been shown to co-express cytokeratins and vimentin (Leong et al., 1988; Viale et al., 1988; Raymond and Leong, 1989; Lauweryns et al., 1993; Ghoubay-Benallaoua et al., 2011). Co-expression of both cytokeratin and vimentin intermediate filaments may occur as a result of epithelial to mesenchymal transition and dual-positive circulating tumour cells have even been identified, the existence of which supports the role of EMT in cancer progression (Barriere et al., 2012; Kasimir-Bauer et al., 2012). Alternatively, there is evidence to suggest that vimentin is expressed by primitive epithelial cell types including stem and progenitor/transit amplifying cells (aka intermediate cells) (Lauweryns et al., 1993; Korsching et al., 2005; Ghoubay-Benallaoua et al., 2011), therefore, vimentin expression in cultured prostate cancer-derived epithelia may indicate the presence of prostate cancer stem/progenitor cells. Further complications are apparent since there are no markers to distinguish between EMT-derived mesenchymal cells and cells of the prostate stroma, hence, it is very difficult to ascertain whether or not primary epithelial cultures present with stromal contamination, as a result of inadequate separation procedures, or evidence of EMT. However, co-expression of cytokeratins and vimentin on fibroblastoid cells may be indicative of EMT since non-EMT-derived stromal cells would not express cytokeratins. Nonetheless, in order to identify the vimentin-positive cells that may present in epithelial cultures, additional mesenchymal markers must be employed.

Morphological differences between cultured epithelial and stromal populations are clearly identifiable microscopically. Epithelial cultures generally present with colonies of small cuboidal cells exhibiting cobblestone morphology whereas stromal cells demonstrate
spindle-like, fibroblastoid morphology (See Figure 1.13) (Peehl, 2002). During this study, microscopic examination suggested that separate epithelial and stromal cultures had been successfully derived from prostate cancer and BPH patient material and in order to confirm this, immunofluorescence was conducted using epithelial (cytokeratins) and mesenchymal (vimentin) markers.

Immunohistochemical testing confirmed that the stromal cultures were single vimentin-positive hence, free from epithelial contamination however, dual vimentin/cytokeratin-positive cells were observed in the epithelial cultures. The co-expression of cytokeratins by several of the vimentin-positive cells in the epithelial cultures ruled out the possibility that these cells were contaminating stromal cells. However, due to the aforementioned problems, using these two markers it was not possible to conclusively confirm whether the dual-positive cells represented primitive cell types or EMT-derived populations.

In a preliminary attempt to distinguish between primitive cells, contaminating stroma and EMT-derived mesenchymal cells, the stromal and epithelial cultures were co-stained with vimentin and fibronectin. This staining revealed that all cells within the stromal cultures co-expressed these markers, hence, it would be fair to assume that any stromal contaminants present in epithelial cultures would co-express vimentin and fibronectin.

However, several studies, including the present study, have revealed that EMT-derived cells may also co-express vimentin and fibronectin. Furthermore, cells which have fully transitioned via EMT have been shown to replace cytokeratins with vimentin, hence would be indistinguishable from stromal/mesenchymal cells using vimentin/cytokeratin staining (Savagner, 2010). In addition, to date, there are no markers which successfully distinguish between normal stromal cells and populations which have fully transitioned via EMT. Therefore, although co-expression of epithelial and mesenchymal markers may identify transitioning cells, cells which have fully transitioned via EMT may be confused with stromal contaminants (Thompson and Newgreen, 2005).

Furthermore, epithelial cells have also been shown to express fibronectin (Kolachala et al., 2007). However, since distinct morphological differences are apparent between epithelial and mesenchymal populations, a combination of marker profile and observation of cellular morphology may be used to distinguish between primitive epithelia and mesenchymal cell types although it is not currently possible to ascertain the source of the mesenchymal/stromal cells.
Interestingly, co-staining with vimentin/fibronectin revealed that there were at least two distinct vimentin-positive populations within the primary malignant epithelial cultures; those which were large, exhibited fibroblastoid morphology and co-expressed fibronectin, and those which were small, non-fibroblastoid and single vimentin-positive. The large, dual-positive, fibroblastoid cells may have been stromal contaminants or cells which had fully transitioned via EMT. Whereas, the lack of the mesenchymal marker, fibronectin, coupled with the small size and epithelial morphology of the other vimentin-positive population, suggested that these cells may have represented primitive cell types such as transit-amplifying/stem cells or epithelial cells which were in the early stages of EMT (Korsching et al., 2005).

It is evident that several markers would be required in order to conclusively identify the distinct vimentin-positive populations within epithelial cell cultures, employing stem cell-associated markers in addition to EMT-associated markers to distinguish between primitive and transitioning cells. Unfortunately, however, immunofluorescence is limited with regards to the number of markers that can be examined. Therefore, while this technique may have provided preliminary evidence of EMT or primitive populations in primary cultures of prostate cancer epithelia in the present study, these data must be supported with further experimental evidence. However, several problems, encountered during the culture of primary prostate epithelia, restricted the interrogation of these populations using other techniques. Such issues included senescence prior to reaching confluence, strong cell-substratum adhesion, which rendered these cells extremely difficult to harvest, and, consequently, sensitivity of these cells to harvesting, which generally resulted in cell clumping and a substantial loss of cell viability. As a consequence, techniques requiring a large number cells, such as flow cytometry, which enables examination of multiple markers, thus, would have proven ideal for the identification of distinct cell types, could not be performed and cells were only examined using immunofluorescence. Moreover, since several cell-surface markers, including the epithelial marker E-Cadherin, are sensitive to harvesting methods, flow cytometric analysis may have generated false profiles. Therefore, a combination of assays would have been required in order to provide substantial evidence of EMT in prostate cancer tissue-derived epithelial cultures.

However, in order to conclusively demonstrate EMT in primary prostate cancer epithelial cells, it would be necessary to work on clonally derived cultures and confirm that single epithelial cells give rise to mixed populations of epithelial and mesenchymal cells. Indeed,
it has long been known that even clonally-derived cultures of primary mammalian epithelia may present with apparent fibroblast overgrowth, as a result of EMT (Zeisberg and Neilson, 2009). As such, single-cell laser-capture-microdissection (LCM) was attempted on single E-Cadherin-positive (epithelial) cells. In addition, cloning of the epithelial cultures by limiting dilution was attempted. Despite several attempts however, it was not possible to clone the primary epithelial cells using either of these techniques. Therefore, studies on primary cells were restricted to mixed (non-clonally-derived) populations that, subsequently, resulted in ambiguous results.

Consequently, it is important to acknowledge that the data presented in this chapter are preliminary and on a limited sample set. Thus, future work, on a larger number of prostate cancer tissue-derived epithelial cultures, using varied techniques, with an extended panel of EMT and CSC-associated markers, is required in order to provide compelling evidence of EMT in primary cultures of prostate cancer epithelia. Furthermore, since the expression of vimentin in human carcinomas has been shown to correlate with invasiveness and metastasis (Takemura, 1994; Singh, 2003; Zeisberg and Neilson, 2009), *in vitro* and *in vivo* invasion and metastasis assays should be conducted on the vimentin-positive primary epithelial cultures in attempt to correlate the EMT-phenotype with characteristics associated with tumour progression.

During this study, cells co-expressing epithelial and mesenchymal markers in prostate cancer epithelial cultures could not be conclusively identified as carcinoma cells transitioning via EMT or cancer stem/progenitor populations. Nonetheless, the observation of multinucleate (i.e. cancer) cells co-expressing epithelial markers (E-Cadherin/cytokeratins) and vimentin in epithelial cultures, arguably provided evidence for either of these phenomena which undoubtedly justified the investigation of cancer stem cells and epithelial to mesenchymal transition in human prostate cancer.

In recent years, the critical involvement of the stromal microenvironment in tumourigenesis has received much attention and tumour associated fibroblasts (aka activated-fibroblasts/myofibroblasts) have been shown to contribute significantly towards cancer growth and progression (Kalluri and Zeisberg, 2006; Pietras and Ostman, 2010; Li et al., 2012; Tripathi et al., 2012). Although the role of EMT in the generation of tumour-associated stroma remains controversial and unproven, carcinoma cells have been shown to be capable of activating EMT *in vitro* and stromal cells adjacent to primary epithelial
tumour nodules have been shown to share some genetic mutations with tumour cells, indicating a contribution of neoplastic cells to stroma formation via EMT (Wernet et al., 2001). Considering these factors, it is not unfeasible that tumours may contribute to their own stromal microenvironment via EMT.

During this study, prostate cancer-derived stromal cultures appeared to proliferate at a high rate and were cultured beyond six passages without senescing whereas, the BPH-derived stromal cultures appeared to grow much slower, often failed to reach confluence and senesced prior to being passaged four times. In light of these observations, it was hypothesised that the stromal cells derived from prostate cancer tissues may contain a population of self-renewing, cancer cells, derived from EMT of the carcinoma cells. Therefore, these cultures were hypothesised to comprise mesenchymal-like cancer cells, hence, possess tumourigenic capabilities. Since ethical constraints prohibited in vivo studies using patient material, to test this hypothesis, a preliminary karyotyping was conducted on one BPH and one PCa-derived stromal population by Dr Karen Sisley at The University of Sheffield. This proof-of-principle analysis was inconclusive, however, it did reveal that changes could be observed therefore, in future a larger sample set, comprising multiple benign and malignant primary cultures, may provide a suitable model to test this hypothesis. The ultimate proof of this concept would be provided by demonstrating in vivo tumour-formation by tumour-derived stromal cells however, until ethical approval is granted, this experiment cannot be conducted.

Although a large body of in vitro evidence, supporting the role of EMT in cancer progression, has been reported using cultured cells from several malignancies, an apparent lack of evidence in clinical specimens has rendered the role of EMT in cancer progression a controversial and disputed topic (Tarin, 2005; Garber, 2008). However, as outlined in Section 1.6.3.6, several explanations may account for the apparent inability to observe EMT in clinical specimens namely; the transient nature of EMT, which may result in it being overlooked, the lack of markers that can distinguish between EMT-derived mesenchymal cells and normal stroma, the possible infrequency of EMT events in vivo and the potential of metastasising EMT cells to revert via MET to an epithelial form, thereby eliminating evidence of the EMT process (Foroni et al., 2012). However, though direct proof of cancer-derived mesenchymal populations in clinical specimens has been difficult to obtain, several studies have correlated the presence of EMT-associated markers/phenotypes, including EMT-inducing transcription factors and Cadherin switching
(from E to N), in clinical specimens with disease progression, relapse and poor survival, thereby, associating the EMT phenotype with tumourigenesis (Hirohashi, 1998; Wang et al., 2004; Kwock et al., 2005; Gravdal et al., 2007).

In an attempt to provide evidence of EMT in clinical specimens, this study examined the presence of E-Cadherin and vimentin in sectioned PCa and BPH tissues. Although vimentin is largely expressed by normal stromal cells, and cells which had fully transitioned via EMT would have been indistinguishable from the normal stromal cells, the co-expression of E-Cadherin and vimentin may have identified cancer cells transitioning via EMT. As previously discussed, co-expression of epithelial markers and vimentin may also identify primitive cells however, since these cells have been reported to constitute rare populations, in both benign and malignant prostate tissues (Collins et al., 2005), an abundance of dual-positive cells would, arguably, be more likely to represent cells activating the EMT programme.

This study revealed potential evidence of EMT in multiple clinical specimens of high-grade prostate cancer, as evidenced by the presence of several cells co-expressing E-Cadherin and vimentin in the cancer specimens but not in sectioned BPH tissues. Furthermore, samples differed in the abundance of co-positive populations, with some samples demonstrating few dual positive cells compared to others in which all E-Cadherin-positive cells co-expressed vimentin. An abundance of EMT-derived populations in high grade prostate cancer tissue specimens would account for the aggressivity associated with this disease pathology as EMTs have been shown to possess enhanced migratory and invasive capacity, hormone resistance and resistance to chemotherapy, all of which are features of advanced castration-resistant hormone-refractory prostate cancer (Kasper and Cookson, 2006; Arumugam et al., 2009; Voulgar and Pintzas, 2009).

Although two markers are not sufficient to conclusively demonstrate EMT, these preliminary data provide evidence of EMT in an in vivo setting which may be verified in future studies using a larger panel of EMT-associated antibodies. One study revealed that the levels of the EMT-inducing transcription factor Twist positively correlated with Gleason Grade and metastasis (Kwok et al., 2005). Therefore, examination of EMT-inducing transcription factors in combination with a larger panel of epithelial/mesenchymal markers in serial sections of prostate cancer tissue may provide more compelling evidence of EMT in vivo.
Previous work identified the OPCT-1 clone, P4B6, as being positive for epithelial to mesenchymal transition (Chapters 4 and 5). Throughout this study, similarities observed between primary cultures of prostate cancer-derived cells and the EMT-positive OPCT-1 clone, P4B6, provided further evidence of EMT in patient material. Not only were morphological similarities observed, in the arrangement of stromal and epithelial cells in primary cultures and in clone P4B6, microscopically, the analogous patterns of mesenchymal cells surrounding epithelial colonies were confirmed by immunofluorescence. Furthermore, primary prostate cancer-derived stromal cultures appeared to reflect the organisation of the mesenchymal cells in the P4B6 clone. Collectively, these data supported the notion that tumours contribute to their own stromal microenvironment via EMT, as a clonally derived population of prostate cancer cells (clone P4B6) not only generated mesenchymal cells, but also organised these cells in such a way as to enable them to perform the functions they would perform in vivo (paracrine feeding of the parenchymal epithelial cells). Furthermore, this finding validates the use of cell lines to investigate the phenomenon of EMT because, despite artificial transformation with HPV genes and several passages in vitro, the clonally derived P4B6 cells were able to organise themselves in the same manner as the primary cells.

In addition to these findings, several other comparable observations were made between clone P4B6 and primary epithelial cultures by immunofluorescence using EMT-associated markers. Perhaps the most noteworthy of which was the observation that cells on the edge of the colonies of the primary prostate cancer-derived epithelia were shown to express vimentin and appeared more spindle-like than those in the centre of the colony. This finding closely resembled the staining of the P4B6 cells following cloning into fluorescence-compatible 96 well plates (See Figure 5.1). Furthermore, this staining pattern provided evidence for the hypothesis that cancer cells on the periphery of the tumour activate the EMT programme, invade and metastasise to distant sites.

In summary, the work embodied in this Chapter provided preliminary evidence of epithelial to mesenchymal transition events in both primary cultures derived from prostate cancer tissue and sectioned prostate cancer tissues. In addition, preliminary evidence to support the hypothesis that tumours contribute towards their own stromal microenvironment via EMT, was also provided. Although this work was preliminary, hence, open to interpretation and scrutiny, these initial findings provide justification for further, extensive studies on patient material. Furthermore, analogous observations
between primary epithelial cultures and the EMT-positive P4B6 clone not only provided evidence of EMT in primary cultures, but also validated the use of prostate cancer cell lines to investigate this phenomenon in human prostate cancer.

Future studies using primary prostate cultures should focus on deriving clones, which should be karyotyped to confirm the benign or malignant cell of origin and exploited as a model to investigate evidence of EMT and the reverse phenomenon MET in non-immortalised primary prostate cultures.
Chapter 8:

Discussion

8.1 Introduction

Prostate cancer represents a major health concern worldwide since, being the third most common cancer in men, it is a significant cause of morbidity and mortality, particularly among men in the developed world (Groenber, 2003; Drewa et al., 2008). Malignancies of the prostate gland account for a quarter of all new cancer cases diagnosed in men in the UK, hence, prostate cancer is the most common male malignancy in the UK (Cancer Research UK, 2012). Although in the case of localised prostate cancer surgery and radiation therapy can prove to be curative, this is not true of the majority of cases, which present with locally advanced or widespread disease. In the case of advanced disease, androgen ablation hormone therapy is the standard first line of palliative treatment. However, despite hormone therapy achieving castrate-levels of testosterone, cancer progression to biochemical or metastatic hormone refractory disease often leads to death since metastatic hormone-refractory prostate cancer (HRPC) currently remains an incurable disease (Hadaschik and Gleave, 2007; Stavridi et al., 2010).

In recent years, the cancer stem cell (CSC) hypothesis has emerged as a model to describe cancer development. Unlike previous models, which proposed that cells within a tumour are equally malignant, the CSC hypothesis postulates that tumours are organised in a hierarchical manner, with only a rare subpopulation of cancer cells (i.e. the CSCs) possessing the unique biological properties required to initiate tumourigenesis (Lawson and Witte, 2007). While there is evidence to suggest that CSCs arise from the malignant transformation of normal tissue stem cells in some cancers (Collins et al., 2005; Merlos-Suárez et al., 2011), the origin of CSCs remains a controversial topic, with several alternative theories having been proposed. Indeed, there may be more than one population of so-called cancer stem cells present within a given tumour, all of which may play a significant role in tumourigenesis (May et al., 2011).
Not only are CSCs believed to be the origin of a given malignancy, hence, the site of malignant transformation and subsequent tumour-initiation, they are also hypothesised to contribute to tumour maintenance, metastasis and resistance to therapy. Moreover, these cells are held accountable for tumour relapse, as it is believed that, akin to normal stem cells, CSCs possess multiple resistance mechanisms and are capable of undergoing long periods of quiescence, which enable them to resist and reside following therapy, while awaiting appropriate microenvironmental signals for the re-initiation of tumour growth, sometimes several years later (Moore and Lyle, 2011). As such, it is believed that current cancer therapies fail to target the resistant CSCs and in order to achieve complete tumour eradication, it is necessary to develop CSC-specific therapies that may be used in combination with conventional therapies to successfully target the bulk of the tumour as well as the CSCs maintaining its growth (Neuzil et al., 2007; Dunning et al., 2011).

Recent advances in stem cell technologies have enabled the identification, isolation and characterisation of putative cancer stem cells from haematological and solid human malignancies including AML, CML, breast, ovarian, pancreatic, colon, brain, head and neck, lung, liver, skin and prostate cancers (Bonnet and Dick, 1997; Singh et al., 2003; Fang et al., 2005; Collins et al., 2005; Szotek et al., 2006; Li et al; 2007; Ricci-Vitiani et al., 2007; Zhao et al., 2008; Visvader and Lindeman, 2008).

In 2005, Collins et al., provided evidence for the stem cell origin of prostate cancer when they isolated putative prostate cancer stem cells using the marker profile they had previously established for normal prostate stem cells (Collins et al., 2005). However, it is important to acknowledge that the origin of prostate cancer still remains an elusive and much debated topic (Maitland et al., 2011).

By exploiting the putative prostate cancer stem cell (PCSC) marker profile and the methods established by Collins and colleagues (2005), this project originally sought to identify, isolate and characterise putative human PCSCs from cell lines and fresh tissue samples, with a view to examining their immunological status, identifying potential expression of cancer-associated antigens and ascertaining whether or not these cells could represent viable targets for immunotherapy. However, perhaps due to the sensitivity of the CD133 molecule, a cell-surface molecule shown to be key for the identification of putative PCSCs, and the limited availability of appropriate antibodies for the recognition of this antigen (problems acknowledged by Maitland et al., 2011), putative prostate cancer stem
cells, with the marker profile CD133+/CD44+/integrin α2β1hi, were neither identified nor isolated using various immunological techniques during this study. Consequently, it was necessary to approach the topic of prostate cancer stem cells from an alternative angle.

Epithelial to mesenchymal transition (EMT) is a vital process which is activated during embryogenesis and throughout life during organ remodelling and tissue repair in response to injury. Recently, EMT has been implicated in the conversion of early stage tumours into invasive, metastatic malignancies. Furthermore, this process is believed to generate cells with the properties of stem cells, thereby bestowing multiple mechanisms of therapeutic resistance including periods of quiescence which may be accountable for tumour recurrence (Mani et al., 2008; Hollier et al., 2009). Since metastasis is accountable for more than 90% of cancer-associated mortalities, the convergence of the EMT and CSC fields was an exciting progression in cancer research as the proposed acquisition of the invasive and migratory mesenchymal traits coupled with the stem-like self-renewal capabilities, bestowed by EMT events, are believed to account for the successful completion of the invasion-metastasis cascade by metastatic cancer cells (Brabletz, 2012).

However, as the EMT and CSC fields remain in their infancy, it is apparent that discrepancies are prevalent within the literature (Kong et al., 2011). For example, although it is evident that the cancer stem-like cells generated via EMT of carcinoma cells (i.e. EMT-derived CSCs) must be distinct from the original tumour-initiating CSCs, while some groups acknowledge that there appear to be more than one type of so-called “cancer stem cell” (May et al., 2011; Chaffer et al., 2011), others reject the original, hierarchal cancer stem cell hypothesis in favour of EMT (Satisteban et al., 2009). Furthermore, it remains to be shown whether EMT events always confer stem-like properties (Brabletz, 2012), and whether or not the ability to activate EMT is restricted to certain populations within a tumour.

To date, much of the research into EMT has been conducted using breast cancer as a model, although evidence pertaining to the significance of this phenomenon in prostate cancer progression is also emerging and the prostate cancer gene fusion TMPRSS2/ERG has even been implicated in the promotion of EMT (Kong et al., 2010; Nauseef et al., 2011; Leshem et al., 2011).

This study aimed to identify a model prostate cancer cell line, showing evidence of spontaneous EMT-events, which could be cloned in order to investigate whether cancer
cells derived from the same tumour exhibit equal potential to activate the EMT programme. Furthermore, this study sought to determine whether or not EMT events always generate cells with the properties of stem cells in a prostate cancer model. Moreover, using these clones, this project aspired to resolve some of the discrepancies in the literature by demonstrating that epithelial to mesenchymal transition and cancer(ous) stem cells are distinct but related phenomena. By creating a model to investigate EMT in human prostate cancer, this project hoped to enable future work on the EMT-derived populations, with a view to determining whether distinct EMT events may give rise to different cell types, such as mesenchymal stem cells and fibroblasts, and whether these cells can be targeted using immunotherapeutic strategies. In addition to cell line studies, this project aimed to provide preliminary evidence of EMT in primary prostate cancer-derived cultures and clinical specimens.

8.2 OPCT-1 is a Model Cell Line for the Investigation of EMT in Human Prostate Cancer

Investigation of epithelial to mesenchymal transitions *in vitro* has previously been achieved in many studies via artificial induction of EMT using one of several methods including stimulation with growth factors such as TGF-β and EGF, forced overexpression of growth factors such as PDGF-D and ectopic expression of EMT-inducing transcription factors such as Snail and Twist (Mani *et al.*, 2008; Kong *et al.*, 2010; Li and Zhou, 2011; Moustakas and Heldin, 2012). This study however, sought to identify a cell line in which spontaneous EMT-events were occurring.

To that end, five prostate cancer cell lines, two derived from metastatic lesions and three from primary tumours, were examined for the expression of several EMT-associated markers (Mani *et al.*, 2008; Zeisberg and Neilson, 2009; Kong *et al.*, 2010). Interestingly, all five cell lines demonstrated expression of mesenchymal proteins however, only the OPCT-1 cell line appeared to present with distinct epithelial and mesenchymal populations. Therefore, the OPCT-1 cell line was selected as a potential model to investigate EMT in human prostate cancer. However, unlike the well characterised PC3 and DU145 cell lines, which have been previously exploited in studies investigating epithelial to mesenchymal transition (Kong *et al.*, 2008; Kong *et al.*, 2009; Kong *et al.*, 2010; Viticchiè *et al.*, 2011), OPCT-1 remains poorly characterised, with very few
publications reporting studies on this cell line (Li et al., 2011). Consequently, prior to conducting further assays, it was necessary to ascertain that the mesenchymal populations had not arisen as a result of stromal contamination (prior to the cell line being immortalised with the HPV16/HPV18 E6 and E7 genes) and rather, that the mesenchymal-like cells represented cells that had transitioned via EMT. Cloning by limiting dilution assays revealed that single OPCT-1 cells gave rise to mixed populations of epithelial and mesenchymal cells, thereby suggesting that the OPCT-1 cell line comprised cells capable of activating the EMT programme. Subsequently, 12 phenotypically distinct OPCT-1 clones, with regards to vimentin expression, were selected and re-screened in order to investigate the stability of their E-Cadherin/vimentin marker-profiles. Interestingly, the majority of the OPCT-1 clones maintained the same proportions of vimentin-positive cells, with some clones expressing very low and others expressing high levels of vimentin. Therefore, these clones provided a suitable model for the characterisation and comparison of vimentin-high and vimentin-low populations, derived from the same prostate cancer cell line.

### 8.3 Clones Derived from the OPCT-1 Cell Line Exhibit Distinct EMT-associated Marker Profiles

Prior to conducting characterisation assays, five of the phenotypically stable OPCT-1 clones (ranging from very low to high in terms of vimentin expression) were selected, seeded at the single cell level into fluorescence-compatible 96 well plates, and examined after a period of growth by immunofluorescence in order to re-confirm that single OPCT-1 cells were capable of generating mixed progeny. Since vimentin expression by carcinomas is widely used as an indicator of Type 3 EMT (Kalluri and Weinberg, 2009), the expression of vimentin, predominantly by cells on the periphery of the colonies, was indicative of EMT in some of the OPCT-1 clones. However, as cancer cells are genetically unstable, aberrant expression of vimentin is not necessarily indicative of EMT. Furthermore, primitive epithelial cell types are also known to express vimentin (Langa et al., 2000) and cancer cells may also de-differentiate, thereby reverting to a more primitive form (Chaffer et al., 2011). Consequently, prior to conducting further characterisation assays, it was necessary to examine the five OPCT-1 clones for the expression of several mesenchymal-associated markers and EMT-inducing transcription factors to conclusively demonstrate evidence of EMT in the vimentin-high clones.
As anticipated, high vimentin expression, determined by flow cytometry in addition to immunofluorescence, correlated with the expression of other EMT-associated markers, examined by IF and Western blotting. Hence, the OPCT-1 clones expressing the highest levels of vimentin appeared to contain the largest populations of mesenchymal cells, as identified by the expression of mesenchymal markers such as N-Cadherin and fibronectin. In contrast, the least vimentin-positive clone demonstrated very low/no levels of all EMT-associated markers, thus, appeared to represent an epithelial-only (EMT-negative) clone.

In summary, the five OPCT-1 clones, selected for further interrogation, were shown to exhibit a range of EMT phenotypes hence, this study revealed that carcinoma cells derived from the same cell line varied considerably in their ability to activate the EMT programme. Therefore, the derivation of clones with a range of EMT phenotypes provided a suitable model for the investigation of epithelial-mesenchymal transition in human prostate cancer.

8.4 EMT Does Not Always Generate Cells with the Properties of Stem Cells

The recent convergence of the emerging EMT and CSC fields by Mani and colleagues, in 2008 provided an explanation for several of the previously unexplained phenomena in cancer biology (Mani et al., 2008). In addition to bestowing apoptotic resistance (Conticello et al., 2004; Liu et al., 2006; Qi et al., 2012) and immune evasion strategies (Wu et al., 2007; Brown et al., 2012; Morandi et al., 2012), the acquisition of stem cell traits by invasive carcinoma cells is believed to account for the enhanced proliferation and self-renewal abilities required for the formation of macroscopic metastases (Brabletz, 2012). Since the discovery by Mani et al., (2008), several other groups have verified a connection between EMT and stemness, particularly in models of breast cancer (Morel et al., 2008; Santisteban et al., 2009; Asiedu et al., 2011; Kasimir-Bauer et al., 2012; Yoon et al., 2012). However, there have also been studies linking epithelial to mesenchymal transition with stem cell signatures in other malignancies including prostate, liver, colorectal and pancreatic cancers (Kong et al., 2010; Dang et al., 2011; Fan et al., 2012; Rhim et al., 2012).

In 2010, Kong et al., artificially induced EMT in the prostate cancer cell line PC3, by forced expression of PDGF-D, and compared the gene signatures of the EMT-induced cells with the original PC3 cell line. Microarray analysis revealed that the expression of the
stem cell-associated transcription factors, Sox2, Nanog and Oct4 was significantly increased in the EMT-induced cells, compared with the original cell line, and this was further validated by RT-PCR and Western blot analysis. In addition, the EMT-induced cells demonstrated stem cell attributes such as increased clonogenic capacity and self-renewal ability (Kong et al., 2010). In an attempt to validate these findings, using a spontaneous model of EMT to investigate a possible correlation between EMT and the generation of stem-like cells by prostate carcinoma cells, this study investigated the protein expression of the prostate cancer stem cell associated markers, integrin α2β1 and CD44, and the stem-cell associated transcription factors, Sox2, Nanog and Oct4, by the five OPCT-1 clones and parental OPCT-1. In addition, several assays, assessing stemness, drug resistance, invasive/migratory capacity and in vivo tumourigenicity were conducted in attempt to ascertain whether or not OPCT-1 clones with increased vimentin-positivity (i.e. EMT) presented with a more aggressive, cancer stem-like phenotype.

Western blot analyses revealed that the most vimentin positive clone (P4B6) expressed the highest levels of the PCSC marker, CD44, in addition to the highest levels of the stem-cell-associated transcription factor, Oct4. However, this clone was not shown to express the highest levels of the additional pluripotency transcription factors, Nanog and Sox2, a finding which was inconsistent with that of Kong et al (2010), who demonstrated that EMT-derived cells expressed significantly higher levels of Nanog and Sox2 than the parental cell line from which they were derived. Interestingly, one of the least vimentin-positive clones (P6D4) was shown to express the highest levels of Nanog. Therefore, the expression of stemness genes was not restricted to clones with a high proportion of EMT-derived cells thus, overall, Western blot analysis failed to demonstrate a direct link between EMT and increased expression of stem-cell associated proteins.

Several assays, conducted throughout the duration of this study, revealed that the most vimentin-positive clone (P4B6) exhibited an aggressive, migratory, cancer-stem-like phenotype. Consistent with this finding, the least vimentin-positive clone (P5B3) demonstrated a non-aggressive phenotype, with limited evidence of stem-like attributes. Taken alone, these two clones conformed to the hypothesis that epithelial to mesenchymal transitions of human prostate cancer cells generate cells with properties of stem cells, however, when all five OPCT-1 clones were taken into consideration, aggressivity and stem-like attributes did not correspond concomitantly with an increase in the percentage of EMT-derived cells.
Paradoxically, in contrast to the most vimentin-positive clone (P4B6), the second most vimentin-positive clone (P2B9) exhibited one of the least aggressive phenotypes overall, with little evidence of stem-like characteristics. As such, it is plausible that the mesenchymal-like cells derived from the EMT events in this clone were distinct from the EMT-derived cells from the most vimentin-positive clone. Therefore, this study provided evidence to suggest that EMT of prostate cancer cells may give rise to distinct progeny hence, epithelial to mesenchymal transitions of human carcinomas may not always generate cells with the properties of stem cells.

The possibility of heterogeneous EMT events may have been overlooked in previous studies, which artificially induced EMT to demonstrate a link between this reprogramming event and the generation of cancer stem cells (Mani et al., 2008; Morel et al., 2008; Santisteban et al., 2009; Kong et al., 2010; Asiedu et al., 2011; Dang et al., 2011; Fan et al., 2012; Kasimir-Bauer et al., 2012; Rhim et al., 2012; Yoon et al., 2012) as, unlike this study, the EMT-positive carcinomas investigated in the aforementioned studies did not comprise mixed populations of epithelia which spontaneously generated mesenchymal-like cells and rather, represented pure populations of (artificially induced) EMT-derived mesenchymal-like cells. Hence, whereas artificial induction of EMT, via ectopic expression of transcription factors, stimulation with growth factors or forced expression of growth factors, may generate cells with the properties of stem cells, naturally occurring EMTs may give rise to distinct cell types in different contextual settings in cancer. Since Type 1 and Type 2 EMT events have been shown to generate mesenchymal cells and fibroblasts, respectively (Kalluri and Weinberg, 2009), it is plausible that cancer cells may also generate distinct cell types via EMT and the results presented in Chapter 6 certainly provide evidence for this hypothesis.

8.5 The OPCT-1 Clones Provide Evidence for the Existence of More Than One Type of Cancer Stem Cell in Human Prostate Cancer

Interrogation of the five OPCT-1 clones (Chapter 6) revealed that clones P4B6 and P6D4 both demonstrated aggressive CSC-associated characteristics. Interestingly, although the clone with the most CSC-like attributes was the most vimentin-positive (P4B6), the second most aggressive/stem-like clone (P6D4) expressed the second lowest levels of the
intermediate filament protein, vimentin. Therefore, as previously stated, vimentin positivity did not always correlate with stemness and aggressivity.

Consistent with previous studies (Mani et al., 2008; Morel et al., 2008; Santisteban et al., 2009; Kong et al., 2010; Asiedu et al., 2011; Dang et al., 2011; Fan et al., 2012; Kasimir-Bauer et al., 2012; Rhim et al., 2012; Yoon et al., 2012), the most vimentin-positive clone (P4B6) also exhibited heightened migratory/invasive capacity and stem-like characteristics, such as enhanced proliferative capacity, superior colony and sphere-forming ability, and elevated ALDH1 activity. Moreover, this clone was the most tumourigenic in vivo, as shown by the formation of tumours that were significantly larger than those formed by the parental cell line from which this clone was derived. Since the cancer stem cell hypothesis postulates that only CSCs possess the unique properties required for tumour initiation (Lawson and Witte, 2007), tumourigenicity is the quintessential feature of cancer stem cells. Therefore, taking all assays into consideration, this clone undoubtedly demonstrated cancer-stem-like attributes hence, was likely to be enriched for a population of (EMT-derived) cancer stem cells.

Intriguingly however, clone P6D4, the second least vimentin-positive clone, also appeared to be enriched for cancer stem cells. This was evidenced by the fact that this clone was by far the most drug resistant of the OPCT-1 clones, a characteristic largely attributed to cancer stem cells (Dean et al., 2005; Zhou et al., 2009; Frank et al., 2010; Borst, 2012), in addition to expressing the highest levels of Nanog and ALDH1, both of which are widely regarded as stem cell and CSC markers (Ginestier et al., 2007; Douville et al., 2009; Jeter et al., 2009; Jiang et al., 2009; Tanei et al., 2009; Jeter et al., 2011). Furthermore, this clone was the second most tumourigenic of the OPCT-1 clones and the histology of the tumours derived from this clone, which appeared to demonstrate the formation of glandular structures, indicated that a stem cell component, capable of differentiating into distinct cell types, may have been present.

Therefore, while the most vimentin-positive OPCT-1 clone and the second least vimentin-positive clone were clearly distinct with regards to EMT-status, they both demonstrated aggressive phenotypes with cancer stem-like attributes. This finding provided evidence for the hypothesis that tumours comprise more than one type of so-called “cancer stem cell”, all of which may contribute significantly towards tumourigenesis. For example, the EMT-
derived CSCs are likely to play a critical role in metastasis whereas other CSCs may be more accountable for therapeutic resistance and subsequent tumour relapse.

Perhaps the most compelling evidence, provided in this study, for the existence of multiple cancer stem cell populations was the discovery that the vimentin-positive (EMT-derived) cells in the OPCT-1 cell line did not express the extracellular matrix receptor integrin α2β1, one of the markers Collins et al., (2005) showed to be highly expressed by putative prostate cancer stem cells. Furthermore, clone P6D4, which seemed to be enriched for cancer stem cells, did not express integrin α2β1.

Therefore, this study provided indirect evidence for the existence of at least three distinct putative prostate cancer stem cell populations; those derived via EMT, the putative CSCs represented in clone P6D4 and the cancer(ous) stem cells reported by Collins and colleagues in 2005.

8.6 Primary Cultures and Clinical Specimens Showed Preliminary Evidence of EMT

Due to the fact that primary cultures are widely regarded as superior models for prostate cancer (Peehl, 2005; Miki and Rhim, 2008), this study aimed to provide evidence of epithelial to mesenchymal transitions in primary prostate cancer-derived epithelial cultures. Furthermore, since there is currently a lack of substantial evidence of EMT in clinical specimens (Foroni et al., 2012), this study sought to identify proof of this phenomenon in frozen prostate cancer tissue sections.

Although it was not necessarily possible to distinguish between cancer stem/progenitor cells, dedifferentiated cancer cells, stromal contamination and EMT-derived cells in some cases, primary cultures of prostate cancer epithelia demonstrating staining patterns analogous to those of the EMT-positive OPCT-1 clone P4B6, provided preliminary evidence of EMT in prostate cancer cells. However, in order to conclusively demonstrate evidence of EMT in primary cultures, the cells must be cloned and examined using a panel of EMT-associated markers, in the same manner by which the OPCT-1 study was conducted.

The demonstration of cells co-expressing epithelial and mesenchymal markers in several clinical specimens of high-grade prostate cancer provided preliminary evidence of EMT
however, sections of BPH tissue failed to demonstrate proof of multiple dual-positive cells. An abundance of dual-positive, potentially EMT-derived, populations in high-grade prostate cancer tissue specimens compared with BPH tissues could arguably account for the aggressivity associated with this disease pathology. This is because EMT-derived cells have been shown to possess enhanced migratory and invasive capacity, hormone resistance and resistance to chemotherapy, all of which are features of advanced castration-resistant hormone-refractory prostate cancer (Kasper and Cookson, 2006; Arumugam et al., 2009; Voulgari and Pintzas, 2009). However, since dedifferentiated cancer cells may also express vimentin, it is important to further characterise the dual vimentin/E-Cadherin-positive cells in the prostate cancer specimens by staining serial sections with a panel of mesenchymal markers and EMT-associated transcription factors.

In summary, difficulties encountered during the culture of primary prostate epithelial cells limited the investigation of these populations, which, consequently, were only examined using immunofluorescence. Using this method, preliminary evidence of EMT was observed in primary prostate cancer cells and tissue sections however, these studies must be expanded using a larger panel of EMT-associated markers in order to conclusively demonstrate evidence of EMT in non-immortalised prostate cancer cells.

8.7 Tumours Contribute to Their Own Microenvironment via EMT

In recent years, the tumour microenvironment has been increasingly recognised as an integral component in tumour initiation, growth and progression (Kalluri and Zeisberg, 2006; Pietras and Ostman, 2010; Li et al., 2012; Tripathi et al., 2012). In fact, transformation of carcinoma cells from a stationary, epithelial cell type to an invasive and migratory mesenchymal cell type via the EMT programme is believed to be activated \textit{in vivo} through secretion of growth factors, such as TGF-β, by the tumour-associated stroma (Polyak and Weinberg, 2009). Although there is some evidence in the literature to suggest that tumours may contribute to their own microenvironment via EMT (Wernert et al., 2000), the general consensus is that tumour cells recruit stromal cells and stimulate local fibroblasts to differentiate into myofibroblasts (aka cancer-associated fibroblasts) to supply them with growth factors and nutrients to support tumourigenesis (Schedin et al., 2004; de Miranda et al., 2011; Kidd et al., 2012).
In vivo, the growth and survival of parenchymal epithelial cells is supported by the stromal compartment, which surrounds the parenchyma and secretes growth factors and extracellular matrix proteins to maintain the epithelial cells (Grossfeld et al., 1998). Incredibly, this relationship was observable in clonally-derived populations of cells from the OPCT-1 prostate cancer cell line, which were shown to activate the EMT-programme to generate mesenchymal cells that surrounded the epithelial colonies in the same manner observed in mixed stromal/epithelial primary cultures. This observation provided compelling evidence to suggest that tumours may be capable of contributing to their own stromal microenvironment hence, tumour-associated stroma may well contain malignant, EMT-derived cells. This hypothesis is supported by the study by Wernert et al., (2000) who demonstrated frequent genetic alterations in non-hereditary, invasive human colon and breast tumour cells and adjacent fibroblastic tumour stroma and revealed that both components can share clonal features.

In addition to observations in clonally-derived populations from the OPCT-1 cell line, evidence for this hypothesis was provided in primary cultures of stromal cells derived from high grade prostate cancer tissue which, not only appeared to proliferate much faster than the stromal cells derived from benign tissues, but were also passaged more than six times without reaching a senescent state. In order to test this hypothesis, preliminary karyotyping was performed on one benign and one cancer-tissue derived population. However, this experiment was inconclusive hence, must be repeated.

In summary, this study provided evidence to suggest that, in addition to activating local stroma and recruiting distant stromal cells to the site of the tumour, tumour cells may be capable of contributing to their own stromal microenvironment via EMT. Intriguingly, a similar concept, differentiation of cancer cells to endothelial cells, has also been shown to contribute to the generation of blood vessel walls during the process of angiogenesis (Ricci-Vitiani et al., 2010). Collectively these data reveal that tumours may represent self-sustaining entities, capable of modelling their own microenvironment to ensure their growth, survival and subsequent dissemination.
8.8 Conclusions

This study created a model for the investigation of epithelial to mesenchymal transition in human prostate cancer and revealed heterogeneity in EMT-events among clonally derived populations of prostate cancer cells, some of which generated cells with the properties of cancer stem cells and others generated cells with a non-aggressive phenotype. Using this model, the present study also provided evidence for the existence of distinct cancer stem cell populations in human prostate cancer, which may play both separate and overlapping roles in prostate cancer tumourigenesis. In addition, this work provided preliminary evidence of EMT in primary cultures and clinical specimens. Finally, based on observations made from both primary stromal cultures and one of the model EMT-positive clones, this study revealed that tumour cells may contribute to their own stromal microenvironment via epithelial to mesenchymal transition.

8.9 Future Work

This study has paved the way for future projects to further characterise epithelial to mesenchymal transition in human prostate cancer and determine the molecular mechanisms which govern the EMT-events that generate cancer stem cells compared to those which generate less aggressive progeny. Furthermore, this project has enabled the investigation of EMT-derived cells with a view to identifying which specific cell types they represent and determining whether these cells can be targeted using immunotherapeutic strategies. In future studies, the OPCT-1 clones may be used as a model to investigate metastatic disease and mesenchymal to epithelial transition (MET) events. In addition, future work should aim to provide further evidence of EMT in clinical specimens and primary epithelial cultures of human prostate cancer.

8.9.1 Molecular Characterisation of the OPCT-1 Clones

Molecular biology currently represents an important area in cancer research and by providing a detailed overview of the molecular changes involved in tumour progression, molecular techniques may consequently lead to a better understanding of the carcinogenesis process and enable the development of new prognostic markers and novel therapeutic targets (Nannini et al., 2009). By simultaneously mapping the expression of thousands of genes in a single tumour sample, gene expression profiling analysis with
microarray technology shows great potential in cancer research (Nannini et al., 2009), enabling the identification of up and down regulated genes which may contribute significantly to tumourigenesis.

Previous studies identified and characterised clones with unique EMT profiles however, the underlying genetic and epigenetic mechanisms which govern the EMT events in these clones remain to be determined. To address the genetic mechanisms underlying the differences between the OPCT-1 clones, four of the previously characterised OPCT-1 clones of interest, the two most and the two least aggressive, and a mesenchymal-only clone, derived from one of these clones, will be analysed using microarray technology. A whole-genome approach, comparing the gene expression profiles of these clones, may identify previously unexplored pathways which may contribute to tumour aggressivity and the cancer-stem cell phenotype and consequently, may represent viable targets for cancer therapies.

8.9.2 Investigating the Identity of the EMT-derived Cells

In 2010, Battula et al., demonstrated that EMT-derived cells exhibited multilineage differentiation potential similar to mesenchymal stem cells (Battula et al., 2010). In addition, this study also demonstrated the ability of EMT-derived cells to home to wounds in vivo, in the same manner as mesenchymal stem cells have been shown to do so. The generation of mesenchymal stem-like cells by EMT of tumour cells could account for several of the phenomena observed in human cancer. Not only are mesenchymal stem cells inherently capable of resisting apoptosis via a number of different mechanisms (Szegezdi et al., 2009; Roodhart et al., 2011; Houthuijzen et al., 2012), these cells also possess multiple immunosuppressive functions, including the expression of growth factors such as TGF-β and the expression and secretion of HLA-G (Nasef et al., 2007; Qi et al., 2012). Furthermore, mesenchymal stem cells possess homing abilities, a feature which may account for why cancer cells often metastasise to the bone (Chapel et al., 2003; Erices et al., 2003; Msouel, et al., 2008; van der Pluijim, 2011). It is also plausible that the cancerous mesenchymal stem-like cells may generate the (cancerous) progeny of these cells, which may include osteoblasts, chondrocytes and adipocytes. Evidence for this hypothesis is prevalent in the literature as abnormal ECM production is one of the defining features of cancer stroma (Hanahan and Weinberg, 2000) and studies have even shown the production of chondrocyte proteins in the mammary gland (Bauer et al., 2010).
Furthermore, microcalcifications can be the early and only presenting signs of breast cancer (Nalawade, 2009) and these may potentially be caused by the aberrant differentiation of EMT-derived mesenchymal stem cell progeny (osteoblasts).

In order to provide evidence for this hypothesis in human prostate cancer cells, future work could involve flow cytometric analysis using a panel of mesenchymal stem cell markers to confirm the presence of putative cancerous mesenchymal stem-like cells in the EMT-positive OPCT-1 clones. Furthermore, the multilineage differentiation potential of the EMT-derived cells, into osteoblasts, chondrocytes and adipocytes, could be investigated using a commercially available kit.

8.9.3 Investigating the Immunological Status of EMT-derived Cells

In order to ascertain whether or not EMT-derived cancer stem cells represent viable targets for immunotherapy, it would be necessary to investigate the immunological status of these cells. Future work could therefore examine the expression of classical HLA class I molecules by EMT-derived cells, in addition to investigating the expression of the non-classical, immunosuppressive molecules such as HLA-G, which has been previously shown to be expressed by mesenchymal stem cells (Nasef et al., 2007), cancer stem cells (Brown et al., 2012) and metastatic bone-marrow infiltrating cells from different tissues (Morandi et al., 2012).

In addition, susceptibility to NK and T-cell mediated cytotoxicity and relative lysis of epithelial versus mesenchymal cancer cells should be investigated and considered when developing a strategy for immunotherapy.

8.9.4 Metastasis Assays with GFP-tagged EMT-derived Cells

In order to prove the theory that, like mesenchymal stem cells, EMT-derived mesenchymal stem-like cells are capable of homing to the bone, an in vivo metastasis assay, using EMT-derived cells transfected with a vimentin-GFP reporter construct, could be conducted in immunocompromised mice. This study would require the successful generation of EMTs stably transfected with the vimentin-GFP reporter construct, followed by injection of the cells into the tail vein of a suitable immunocompromised mouse model. The migration of these cells could potentially be monitored using live-animal imaging technology and following completion of the assay, the bone marrow cells could be harvested and examined.
for the presence of GFP-positive cells using fluorescence microscopy. This assay may provide an explanation for the frequent occurrence of bone metastases in breast and prostate carcinomas (van der Pluijim, 2011).

8.9.5 Investigation of Mesenchymal to Epithelial Transition (MET)

Plasticity of tumour cells is one of the traits currently believed to be critical in cancer metastasis. So-called “plasticity type I metastasis” is believed to be characterised by transient EMT-MET processes, whereas “genetic type II metastasis” is characterised by a permanent switch from an epithelial to a mesenchymal state (Brabletz, 2012). Though there is much evidence to support the critical role of epithelial to mesenchymal transitions in tumourigenesis, there is less evidence of the reverse process, mesenchymal to epithelial transition (MET). In an attempt to address this and provide potential evidence of MET events by prostate cancer cells, clone P4B6 B, a mesenchymal-only clone, clonally derived from the EMT-high clone P4B6, could be cloned at single-cell density into fluorescence-compatible 96-well plates using a high-speed cell sorter, and stained for epithelial and mesenchymal markers after a period of growth. The presence of cells expressing epithelial markers would provide in vitro evidence for the occurrence of MET. In addition, evidence of MET events could be investigated in vivo by injecting the mesenchymal-only clone into immunocompromised mice and subsequently excising, sectioning and staining the tumours for the expression of epithelial and mesenchymal markers.

8.9.6 Further Work on Patient Tissue

Work conducted in this study provided only preliminary evidence of EMT in prostate cancer patient samples. Consequently, it is necessary to expand the number of tissues examined and repeat the aforementioned procedures, this time using a larger panel of EMT-associated antibodies. In addition, clonally-derived populations must be established from single primary prostate cancer cells in order to rule out the possibility of stromal contamination affecting the interpretation of the results.

Due to the difficulties encountered with the culture of the primary prostate cancer epithelia, it may be necessary to immortalise these cells using a three plasmid hTERT system. Indeed, permission for the use of this construct to generate “in house” cell lines from the cultured prostate cancer epithelia was sought and approved at Nottingham Trent University. However, since immortalisation would undoubtedly affect the biological
properties of these cells, this procedure would only be necessary if the problems with culturing primary prostate cancer epithelia could not be overcome.

In addition to studies with the tissue sections and epithelial cultures, the stromal cultures derived from prostate cancer tissue should also be karyotyped, along with benign controls. Furthermore, the tumourigenic potential of these cells should also be investigated in an in vivo murine model. These experiments would provide conclusive evidence for the hypothesis that tumours contribute to their own stromal microenvironment.

8.10 Final remarks

In summary, this study generated a model that has enabled investigation into the properties of epithelial to mesenchymal transition and cancer stem cells in human prostate cancer cells. In addition, this project established primary cell culture techniques and provided preliminary evidence of epithelial to mesenchymal transition in primary cultures of prostate cancer epithelia and clinical specimens. The foundations laid by the completion of this project have provided considerable scope for future studies which may identify the cell types generated by distinct EMT events, examine the immunological status and metastatic capability of EMT-derived cells, investigate evidence of mesenchymal to epithelial transition and determine the underlying mechanisms governing distinct EMT events. Taken together, these data provide exciting insights into the fundamental mechanisms involved in prostate cancer progression.
Appendix I

**Information pertaining to the derivation and characterisation of the OPCT cell lines used in this study.**

Derivation and initial characterization of three new prostate cancer cell lines and three new immortalized normal prostate epithelial cell lines

[http://aacrmeetingabstracts.org/cgi/content/abstract/2004/1/183-a](http://aacrmeetingabstracts.org/cgi/content/abstract/2004/1/183-a)

Characterization of three pairs of prostate cells lines derived from tumor and adjacent normal tissues

[http://aacrmeetingabstracts.org/cgi/content/abstract/2005/1/462](http://aacrmeetingabstracts.org/cgi/content/abstract/2005/1/462)

Comparison of gene expression profiles in cell line pairs derived from tumor and adjacent normal prostate tissues

[http://www.aacrmeetingabstracts.org/cgi/content/meeting_abstract/2007/1_Annual_Meeting/1186?maxtoshow=&hits=10&RESULTFORMAT=&searchid=1&FIRSTINDEX=0&minscore=5000&resourcetype=HWCIT](http://www.aacrmeetingabstracts.org/cgi/content/meeting_abstract/2007/1_Annual_Meeting/1186?maxtoshow=&hits=10&RESULTFORMAT=&searchid=1&FIRSTINDEX=0&minscore=5000&resourcetype=HWCIT)

**OPCT Cell line information**

**OPCT-1**


**OPCT-2**

## Appendix II

The full list of equipment used throughout the duration of this study.

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<th>Equipment</th>
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<td>Beckman Coulter</td>
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Nanopure Diamond water reservoir
Nikon Eclipse Ts100 Light Microscope
NXT Top-Count Microplate Scintillation Counter
Octo MACS magnet
Olympus BX51 Fluorescence Microscope
Orbital Incubator
pH meter
Plate reader
Plate rocker
96-well plate harvester
PCR workstation cabinet
Power packs
Real-time qPCR thermal cycler
Refrigerated Centrifuge
Refrigerated Centrifuge 5702 R (4°C)
Refrigerator
Refrigerator
Rocking platform
Sonicator
Sealing tape (Nescofilm)
Tecan Ultra Microtiter Plate Reader
Transfer apparatus
37°C/5%CO₂ incubators
Top count scintillation counter
UNO-thermoblock
Vortex mixer
Water Bath
Weighing scale
Whirlimixer

Barnstead
Olympus
Perkin Elmer
Miltenyi
Olympus
Stuart
Mettler Toledo
Biorad and Tecan
VWR
Packard
Grant-Bio
Biorad
Qiagen
Mistral 1000, MSE
Eppendorf
Lec refrigeration PLC, England
BOROLABS, UK
VWR
VWR
Bando Chemicals
Tecan
Geneflow
Forma Scientific
Packard
Biometra
Scientific Industries
Grant Instruments
Fisherbrand
Scientific Industries
Appendix III

Isotype control staining of PC3, DU145 and P4E6 cells

**Figure A3.1:** Dual immunofluorescent isotype control staining of PC3 cells. Secondary antibody and nuclear staining were achieved as described in Figure 3.1a. (n=3). Representative images. Image magnification at x20.

**Figure A3.2:** Dual immunofluorescent isotype control staining of DU145 cells. Secondary antibody and nuclear staining were achieved as described in Figure 3.1a. (n=3). Representative images. Image magnification at x20.

**Figure A3.3:** Dual immunofluorescent isotype control staining of P4E6 cells. Secondary antibody and nuclear staining were achieved as described in Figure 3.1a. (n=3). Representative images. Image magnification at x20.
Appendix IV

Demonstrating the 12 OPCT-1 clones of interest growing as single-colonies after 21 days in culture following cloning by limiting dilution.

*Figure A4.1:* Demonstrating representative images of the individual colonies formed by the 12 OPCT-1 clones of interest after 21 days in culture. x10 and x20 magnification.
Dual-isotype control staining of 5 prostate cancer cell lines

<table>
<thead>
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<td>OPCT-2</td>
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**Figure A4.2:** Murine and rabbit isotype control staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 cells. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=3). Representative images. Image magnification at x20.
Figure A4.3: Goat isotype control staining of DU145, PC3, P4E6, OPCT-1 and OPCT-2 cells. Secondary antibody and nuclear staining were performed as previously described in Figure 4.2. (n=2) Representative images. Image magnification at x20.
Appendix V

Negative staining of five OPCT-1 clones stained with 3 stem cell associated transcription factors.

Nanog, a homeodomain-containing transcription factor, is essential for the maintenance of pluripotency and self-renewal in embryonic stem cells (Kim et al., 2008). Furthermore, the expression of this protein has been detected in putative cancer stem cells from cancers including prostate cancer (Gong et al., 2012; Shan et al., 2012). Figure A5.1 demonstrates that, using this technique, Nanog was not detected in any of the OPCT-1 clones.

The transcription factor Oct4 is another key regulator of pluripotency and has been shown to be expressed by CSC-like cells in different cancers (Kim et al., 2008; Kumar et al., 2012). Figure A5.2 illustrates that this protein was not detected by immunofluorescence on any of the OPCT-1 clones.

Along with Nanog and Oct4, Sox2 is an essential regulator of pluripotency and as such, is associated with uncommitted dividing stem and precursor cells (Avilion et al., 2003). As with Nanog and Oct4, Sox2 was not detected in any of the OPCT-1 clones by immunofluorescence (Figure A5.3).

Positive control staining of NTERA2/D1

NTERA2/D1 is a pluripotent embryonal carcinoma cell line which does not require feeder-cells to maintain its undifferentiated potential (Simões and Ramos, 2007). As such, it was utilised in this study as a positive control for the pluripotency markers Nanog, Oct4 and Sox2. Figure A5.4 demonstrates that the Nanog, Oct4 and Sox2 antibodies used in this study were suitable for the detection of these transcription factors by immunofluorescence.

Isotype control staining of five OPCT-1 clones

Figures A5.5 and A5.6 demonstrate that minimal background staining was observed with isotype control antibodies and that such background staining was distinct from the staining patterns achieved using the test antibodies.
Figure A5.1: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9, and P4B6 using a rabbit monoclonal antibody against Nanog. Secondary antibody labelling was achieved using an anti-rabbit AlexaFluor®488-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Figure A5.2: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9, and P4B6 using a rabbit polyclonal antibody against Oct4. Secondary antibody labelling was achieved using an anti-rabbit AlexaFluor®488-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Figure A5.3: Immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9, and P4B6 using a rabbit monoclonal antibody against Sox2. Secondary antibody labelling was achieved using an anti-rabbit AlexaFluor®488-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Immunofluorescent staining of the pluripotent embryonal carcinoma cell line NTERA2/D1 as a positive control for the expression of Nanog, Oct4 and Sox2. Secondary antibody labelling was achieved using an anti-rabbit AlexaFluor®488-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=2). Representative images. Image magnification at x20.
Figure A5.5: Murine and rabbit isotype control immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6. Secondary antibody and nuclear staining were performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Figure A5.6: Goat isotype control immunofluorescent staining of clones P5B3, P6D4, P5F3, P2B9 and P4B6. Secondary antibody labelling was achieved using an anti-goat Alexa Fluor®568-conjugated secondary antibody and nuclear staining was performed as previously described in Figure 5.1. (n=3). Representative images. Image magnification at x20.
Appendix VI

Isotype control staining of tumours derived from four OPCT-1 clones and parental OPCT-1.

**Figure A6.1:** Mouse and rabbit isotype control immunofluorescent staining of tumour sections derived from clone P6D4, P2B9, P4B6 and parental OPCT-1. Secondary antibody labelling was achieved using an anti-mouse Alexa Fluor®568-conjugated secondary antibody and an anti-rabbit Alexa Fluor®488-conjugated secondary antibody. Nuclear staining was achieved using mounting media with DAPI. (n=2). Representative images. Image magnification at x10, x20, x40.
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Immunotherapy of prostate cancer: should we be targeting stem cells and EMT?

Naomi L. Dunning · Stéphanie A. Laversin · Amanda K. Miles · Robert C. Rees

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Abstract Cancer stem cells have been implicated in a number of solid malignancies including prostate cancer. In the case of localised prostate cancer, patients are often treated with surgery (radical prostatectomy) and/or radiotherapy. However, disease recurrence is an issue in about 30% of patients, who will then go on to receive hormone ablation therapy. Hormone ablation therapy is often palliative in a vast proportion of individuals, and for hormone-refractory patients, there are several immunotherapies targeting a number of prostate tumour antigens which are currently in development. However, clinical responses in this setting are inconsistent, and it is believed that the failure to achieve full and permanent tumour eradication is due to a small, resistant population of cells known as ‘cancer stem cells’ (CSCs). The stochastic and clonal evolution models are among several models used to describe cancer development. The general consensus is that cancer may arise in any cell as a result of genetic mutations in oncogenes and tumour suppressor genes, which consequently result in uncontrolled cell growth. The cancer stem cell theory, however, challenges previous opinion and proposes that like normal tissues, tumours are hierarchical and only the rare subpopulation of cells at the top of the hierarchy possess the biological properties required to initiate tumourogenesis. Furthermore, where most cancer models infer that every cell within a tumour is equally malignant, i.e. equally capable of reconstituting new tumours, the cancer stem cell theory suggests that only the rare cancer stem cell component possess tumour-initiating capabilities. Hence, according to this model, cancer stem cells are implicated in both tumour initiation and progression. In recent years, the role of epithelial–mesenchymal transition (EMT) in the advancement of prostate cancer has become apparent. Therefore, CSCs and EMT are both likely to play critical roles in prostate cancer tumourigenesis. This review summarises the current immunotherapeutic strategies targeting prostate tumour antigens taking into account the need to consider treatments that target cancer stem cells and cells involved in epithelial–mesenchymal transition.

Keywords Cancer stem cells · Prostate cancer · Prostate tumour antigens · Immunotherapy · EMT · PIVAC 10

Prostate cancer, tumour antigens and their role in immunotherapy

Prostate cancer constitutes a major health problem worldwide since it is a significant cause of morbidity and mortality in men, particularly in the developed world [1]. It is the third most common cancer in men worldwide and is most prevalent in European and North American men [2]. Prostate cancer accounts for a quarter of all new cancer cases diagnosed in men in the UK; hence, it is the most common male malignancy in the UK. Although in the case
of localised prostate cancer, surgery and radiation therapy can prove to be curative, this is not true of the majority of cases which present with locally advanced or widespread disease. In the case of advanced disease, androgen ablation hormone therapy is the standard first-line palliative treatment. However, despite hormone therapy achieving castrate levels of testosterone, cancer progression to biochemical or metastatic hormone-refractory disease often leads to death since metastatic hormone-refractory prostate cancer (HRPC) currently remains an incurable disease [3, 4]. There is an ongoing need to develop new, more accurate screening tests for prostate cancer so that the disease can be identified early on where the treatment options are much more effective. For those hormone-refractory patients, new treatment options are required to help eradicate and/or manage their disease, and to this end, immunotherapy is one line of attack for advanced disease.

In the last two decades, advances in tumour immunology have led to the discovery of many peptides from tumour antigens that are recognised by cytotoxic T lymphocytes [5, 6]. There are two main adoptive immunotherapeutic approaches: undefined antigen-based (whole tumour immunome) therapies and the specific antigen-based therapies [7]. The first approach does not allow monitoring the anticancer immune response to therapy because of the lack of information on the recognised antigen(s), whereas the activity of antigen-specific T cells (CD8+/CD4+) can be assessed when using specific antigen-based immunotherapies [8]. The criteria for a tumour antigen to be a quintessential target for immunotherapy include its specificity to cancer cells, its high expression in cancer cells, its key role in cancer progression, its high frequency in patients, its location on the cell surface or its ability to be efficiently presented on major histocompatibility complex (MHC) molecules and its ability to induce a specific, potent and lasting immune response.

In order to combat the phenomenon of tumour immunoeediting, vaccines should include inhibitors of the activity of regulatory cells (Treg) [9, 10], adjuvants such as toll-like receptor (TLR) agonists (e.g. CpG oligodeoxynucleotides that bind to TLR9) to activate the immune cells [11] and T cells with receptors that have been genetically engineered to be specific to the antigen(s) of interest [12, 13]. Also, antibody-based therapy, radiotherapy, hormonal ablation therapy, chemotherapy or anti-angiogenic therapy should be used in conjunction with the vaccine to increase the death or at least enhance the antigen-presenting capability of cancer cells as well as removing suppressor cells [14–16]. Finally, chemotherapeutic agents can help to increase cytotoxic T-cell entry into the site of the tumour by disrupting the tumour stromal cells [17]. Treating patients with immunotherapy as an adjuvant therapy at earlier disease stages will improve clinical benefits when compared with the vaccination of patients already bearing high-grade tumours [18–20].

Prostate cancer is a promising tumour for targeted treatment using vaccine approaches due to the expression of unique prostate-associated antigens [21]. Tumour antigens were first identified in the early 1970s by Abelev et al. (alpha-fetoprotein) [22] and Gold and Freedman (carcino-embryonic antigen) [23]. Over the last 20 years, the family of tumour antigens identified has vastly increased to include antigen categories such as tumour-associated antigens [24, 25], splice-variant antigens [26], antigens encoded by mutated genes [27], cancer-related autoantigens [28], fusion proteins [29] and, the most promising category of all, cancer testis antigens [30]. Table 1 outlines some of the more promising prostate tumour antigens that have been taken forward into clinical trials in prostate cancer.

Dendritic cells are known to play an essential role in the establishment of both innate and adaptive antitumour immune responses. Dendritic cell-based vaccines have already shown promising results by producing significant immune responses against prostate tumour antigens such as PAP [31, 32], PSMA [33–35] and PSA [36] as well as being essentially free of side effects [37]. For example, a dendritic cell-based multi-epitope vaccine using prostate stem cell antigen (PSCA14–22), prostatic acid phosphatase (PAP299–307), prostate-specific membrane antigen (PSMA4–12) and prostate-specific antigen (PSA154–163) has elicited significant cytotoxic T-cell responses and the generation of memory T cells [38]. Of particular significance, and an important landmark for the treatment of asymptomatic or minimally symptomatic, metastatic HRPC, was the formulation of the first adoptive cell transfer regimen, called sipuleucel-T (PROVENGE), which was approved by the United States Food and Drug Administration in April 2010 [16]. Sipuleucel-T is composed of autologous dendritic cells which are harvested by leukapheresis and processed in vitro in order to express recombinant PAP (PA2024) linked to GM-CSF. The antigen-presenting cells are then intravenously injected to the patient within 2 days and the regimen is given 3 times in total, once every 2 weeks [16]. Patients participating in late-stage randomised trials had a statistically significant extension of their life of at least 4 months, with an overall survival of about 20 months ($P = 0.01$) [39]. The treatment was deemed safe and the most common side effects were fever, tremor, rigours and hypersensitivity to cold. Antibody and T cell-specific activities to PA2024 were observed in the treated patient population. A large phase III study was then conducted with 512 patients, and the data confirmed the preliminary findings and also showed that treating this patient population with sipuleucel-T significantly reduced the risk of death by 22% when compared with placebo ($P = 0.032$) [21]. However, PROVENGE is a
Some of the identified prostate tumour antigens have also been investigated as potential disease biomarkers. Alpha-methyl acyl-CoA racemase (AMACR) is routinely used in combination with p63 on ambiguous clinical prostate cancer biopsies, and trace amounts of autoantibodies against AMACR have been detected in the circulation of prostate cancer patients [44, 45]. Other prostate-related proteins have been included in urine-based diagnostic tests, and these include differential display code antigen 3 [46] and glutathione s-transferase P1 (GSTP1) [47]. In addition, several prognostic and diagnostic markers have been identified, and these include EZH2, a biomarker for post-prostatectomy relapse [48, 49], human kallikrein 2 [50] and early prostate cancer antigen which has been incorporated into an ELISA measuring serum levels for prostate cancer diagnosis [51]. Of particular interest to our group is the prostate-associated tumour antigen termed T21 [52]. T21 was originally identified via the serological screening of a testis cDNA library with sera from prostate cancer patients. The technique of identification alone strongly demonstrates that the immune system of patients with prostate cancer is able to illicit an immune response against T21. Further work on T21 has demonstrated its low-level expression in normal tissues at both the mRNA level and protein level. In prostate cancer, we found T21 mRNA to be significantly over-expressed in malignant glands compared with benign glands and stroma. At the protein level, T21 expression was higher in patients with a high Gleason score, and it was significantly over-expressed in patients with stage pT4 disease. These findings suggest that T21 is a promising marker for late-stage disease and may also prove to be a valuable target for prostate cancer immunotherapy.

### Table 1 Clinically relevant studies utilising prostate-associated antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Function</th>
<th>Clinical studies</th>
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<tr>
<td>Prostate-specific antigen (PSA)</td>
<td>A 34-kDa kallikrein protein with serine protease activity [53]. Routinely used to assess disease progression in prostate cancer patients. Also expressed by cells in the salivary gland, small intestine, smooth muscle and renal microtubules [54]</td>
<td>PROSTVAC—a recombinant vaccinia-PSA [55]. Dendritophage-rPSA—autologous DCs pulsed with recombinant human PSA protein [56]. PSA-TRICORM—a recombinant pox virus vector containing PSA and 3 human T-cell co-stimulatory molecules [57, 58]. DCs loaded with PSA peptides [38, 59] or transfected with PSA mRNA [36]. Co-delivery of PSA and PSMA DNA vaccines using electroporation [60]</td>
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<td>Prostate-specific membrane antigen (PSMA)</td>
<td>A 110-kDa membrane-bound glycoprotein expressed on the surface of prostatic epithelial cells. Its expression has been shown to increase after androgen deprivation therapy [61, 62], and higher levels are correlated with disease stage and Gleason score [61, 63]</td>
<td>Prost30—PSMA directed monoclonal antibody [64, 65]. DCvax—autologous cells exposed to 2 PSMA peptides [34]. DCs loaded with PSMA peptides [33, 54, 66, 67]</td>
</tr>
<tr>
<td>Prostatic acid phosphatase (PAP)</td>
<td>A 386-amino acid protein that is secreted by the prostate [68]. Increased levels of PAP have been detected in the circulation of patients with advanced stage disease [69]. Decreases in PAP levels have been correlated with response to therapy [70]</td>
<td>PROVENGE—a DNA vaccine consisting of a combination of PAP and GM-CSF introduced ex vivo into autologous DC before reinjection into the patient [31]</td>
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<tr>
<td>Prostate stem cell antigen (PSCA)</td>
<td>A GPI-anchored cell surface antigen related to the LY-6/Th-1 superfamily with 30 % homology to stem cell antigen 2 [71]. PSCA is localised to the basal cell epithelium in normal prostates and in prostate cancer it is highly expressed on the secretory epithelium. The acquisition of androgen independence and metastatic disease leads to elevated expression [72–74]</td>
<td>DCs loaded with PSCA peptides administered to hormone and chemo-resistant patients [75]. DCs loaded with a combination of PAP, PSMA and PSA peptides administered to hormone-resistant patients [38]</td>
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A good example of the current limitations of cancer vaccines as it very complex and expensive to produce and only reduces the rate of disease progression rather than curing the cancer [39]. It could be argued that earlier intervention with such forms of immunotherapy would provide longer-lasting clinical benefits.

There are also other prostate antigens that are still in early development and have only been tested in mouse models of prostate cancer. These include six-transmembrane epithelial antigen of the prostate (STEAP), which demonstrated the inhibition of prostate cancer progression when TRAMP-C1 mice were immunised with recombinant DNA and Ankara vectors delivering PSCA and STEAP antigens [40]. Monoclonal antibodies against STEAP-1 significantly inhibited tumour growth in mouse models using patient-derived LAPC-9 prostate cancer xenografts [41]. Prostate secretory protein of 94 amino acids (PSP-94) has also demonstrated efficacy in mouse models; syngeneic models of rat prostate cancer were given either subcutaneous or intracardiac injections of MatLyLu-PTHrP cells, and administration of PSP-94 resulted in tumour growth inhibition and prevention of skeletal metastases [42, 43].
Cell types of the prostate

The three most abundant epithelial cell types within the normal, adult prostate are the secretory luminal, basal and neuroendocrine cells, respectively. The androgen-dependent secretory luminal cells are terminally differentiated and secrete prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) into the glandular lumina in response to androgens. Basal cells, which are not dependent on androgens for their survival and are less differentiated than luminal cells, surround the luminal cells and rest on the basement membrane. The neuroendocrine cells, which are relatively rare in comparison with the other cell types, are terminally differentiated and androgen-insensitive. These cells are involved in the production of neuropeptides which include serotonin and calcitonin. All three cell types are thought to derive from prostate epithelial stem cells which reside in the basal cell compartment [53, 54].

Of late, there has been much evidence implicating epithelial prostate stem cells as targets for transformation in prostate cancer, and this has been the topic of several well-written reviews and research articles [53–59]. Indeed, the role of the so-called cancer stem cells in prostate cancer initiation and progression has sparked huge interest, and, as such, prostate cancer stem cells are the subject of intense investigation.

Cancer stem cells

Cancer stem cells and their potential role in tumourigenesis have been the topic of much debate in recent years as they may help to elucidate some of the unexplained phenomena, including cancer relapse and metastasis. Recent advances in stem cell biology have led to the identification of adult tissue stem cells in various organs which, in turn, has enabled research into such populations as potential candidates for malignant transformation. To date, putative cancer stem cells have been identified and isolated from solid and haematological malignancies including prostate, pancreatic, brain, colon, head and neck, gastric, lung, liver, acute myeloid leukaemia, multiple myeloma, melanoma and ovarian cancers [60–73] (Table 2).

The cancer stem cell is hypothesised to be the original cell of a tumour which is held solely responsible for tumourigenesis, tumour differentiation, tumour maintenance,
tumour spread (metastasis) and tumour relapse following therapy [74]. Cancer stem cells have been shown to demonstrate a marked capacity for proliferation, self-renewal and differentiation and are generally believed to constitute a very rare population of cells among a majority of tumour cells with a more differentiated phenotype. Aside from their self-renewal and differentiation capabilities, cancer stem cells have been shown to share many other properties with adult stem cells. For example, akin to normal stem cells, cancer stem cells are reported to be highly resistant to drugs and toxins, through the expression of drug efflux pumps and are to resist to apoptosis, through a variety of mechanisms including active DNA repair [75–83].

Considering these facts, the proposed properties of cancer stem cells may explain why disease relapses occur in many cancers since it is believed that current cancer therapies only eliminate the bulk of the tumour, leaving the (resistant) cancer stem cells behind to reinitiate tumour growth [84]. Cancer stem cells have been reported to express many genes known to be important in somatic (normal) stem cell maintenance such as BMI-1 and Oct3/4 [85]. Furthermore, many of the markers known to identify putative CSCs in several tumour types are common to the normal adult stem cells from the healthy tissue [60, 86–88]. Such findings support the notion that cancer stem cells are in fact transformed adult stem cells. However, although the cancer stem cell theory draws parallels between normal tissue stem cells and tumour-initiating cells, thus far, not all studies have addressed whether or not cancer arises from the transformation of normal stem cells and have rather suggested that cancers consist of a heterogeneous population of cells which may be organised in a hierarchical manner much like normal tissues [89]. Hence, tumours are thought to contain a stem-like component which drives proliferation although the source of such a component is controversial, with more than one theory for the origin of cancer stem cells having been proposed.

Figure 1 demonstrates that CSCs could potentially arise from mutated adult tissue stem cells, mutated progenitor cell/transit amplifying cells or differentiated cells which acquire mutations conferring de novo self-renewal and differentiation capabilities. Although the origin of the cancer stem cell has not yet been elucidated for every tumour type, there is evidence to support all three mechanisms and perhaps CSCs can arise by any one, or even all, of the three proposed pathways.

There is a large body of evidence to suggest that cancer stem cells are derived from transformed adult tissue stem cells [58, 90–94]. It has been suggested that normal stem cells may be the only cells which live long enough to acquire sufficient genetic mutations to become cancer stem cells [95]. Alternatively, there is also much evidence to suggest that CSCs derive from a more committed progenitor/transit amplifying cell [90, 92, 94] but there is less data to support the notion that cancer stem cells arise from transformed differentiated cells which acquire stem-like properties. However, since it has been proven that differentiated cells can be reprogrammed to induce pluripotency [96, 97], it is plausible that activating mutations in otherwise silenced stem cell genes such as Oct3/4, Sox2, and c-Myc may confer stem-like properties in differentiated cells.

**Stem cells in prostate cancer and EMT**

The stem cell model for prostate epithelia was established by Isaacs and Coffey (1989) following a series of androgen cycling experiments in a rodent model. In demonstrating that the prostate, an androgen-dependent organ, could completely regenerate following castration once androgen levels were restored, they proposed that androgen-independent stem cells yield a population of androgen-responsive cells which in turn give rise to the more differentiated androgen-dependent secretory luminal cells. This model for the hierarchy of the prostate gland is now widely accepted and prostate stem cells have since been isolated and investigated [87] Fig. 2.

In 2005, Collins et al. isolated ‘tumourigenic prostate cancer stem cells’ using the same markers they had previously established for normal prostate tissue stem cells [58, 87]. The marker profile identified for prostate cancer stem cells was integrin$\alpha_2\beta_1$high/CD44+/CD133+. Integrin$\alpha_2\beta_1$ (also referred to as VLA-2) is a transmembrane receptor for extracellular matrix proteins such as laminin, collagen and fibronectin and adhesion molecules such as E-cadherin. Among other functions, it is known to play a role in the generation and organisation of extracellular matrix proteins and mediate interactions between adhesion molecules on adjacent cells. CD44, a cell surface glycoprotein involved in cell–cell interactions, cell adhesion, migration and lymphocyte activation, has been implicated as a cancer stem cell marker for several other cancers including breast, head and neck and colorectal [98–100]. CD133 has also been implicated as a marker for putative cancer stem cells in other cancers including brain, pancreatic and lung [72, 101, 102]. To date, the function of this molecule remains to be elucidated.

Collins et al. [58] demonstrated that cells which were positive for all three markers possessed significant capacity for self-renewal, differentiation and invasiveness in vitro, all properties which have been attributed to cancer stem cells. Such cells may be responsible for both the initiation and progression of prostate cancer, i.e. relapse and metastatic spread despite androgen ablation or other methods. However, this may not necessarily be the case.
Recently, there has been much interest in the phenomenon referred to as epithelial to mesenchymal transition (EMT) and its role in cancer progression and metastasis. Epithelial to mesenchymal transition is a crucial transdifferentiation programme which is known to occur during embryogenesis and in adult tissues following wound repair and organ remodelling in response to injury [103]. During the EMT process, epithelial cells undergo multiple biochemical changes involving the down-regulation of epithelial markers, which confer cell–cell and cell–extracellular matrix (ECM) adhesion, and the up-regulation of mesenchymal markers, conferring increased production of ECM components, enhanced migratory capacity, invasiveness and increased resistance to apoptosis [104, 105]. The hallmarks of an EMT are also characteristic of metastatic cancer cells, as such EMT has recently been implicated in cancer metastasis as the enhanced migratory capacity and invasiveness of mesenchymal cells is believed to facilitate the dissemination of cancer [106]. Furthermore, since EMT has been shown to confer increased resistance to apoptotic...
agents commonly used in chemotherapy, this phenomenon is thought to be a critical step in tumour metastasis [107–109]. In order to establish new tumours at the metastatic sites, it is believed that the cells which transition from an epithelial to a mesenchymal state and migrate must undergo the reverse procedure, mesenchymal to epithelial transition (MET) [110]. Therefore, metastasis is considered to be a dynamic and complex process involving cellular plasticity.

One study conducted in 2005 demonstrated that the expression levels of Twist, a key transcription factor in EMT, were positively correlated with Gleason grading and metastasis [111]. Furthermore, Twist has been shown to inhibit apoptosis in cancer cells and it is thought to play a central role in the resistance to microtubule-disrupting agents [112]. Indeed, the potential of Twist as a target in advanced and/or metastatic prostate cancer has been discussed [105]. In addition, other studies have correlated the presence of critical EMT drivers with disease relapse and poor survival [113, 114]. A recent study, conducted by Tanaka and colleagues, demonstrated a clear link between the expression of N-cadherin, a mesenchymal cadherin associated with EMT, and invasive, metastatic, castration-resistant prostate cancer (CRPC). In this study, N-cadherin-specific antibodies were shown to delay the progression to castration resistance, inhibit the invasion of surrounding tissues, suppress tumour growth and reduce metastasis in castrated mice. Hence, this work provides further support for the critical role of EMT in prostate cancer progression and the potential of immunotherapy as a strategy to combat this disease [115].

In 2008, Mani et al. [116] established a link between EMT and epithelial stem cells in that the products of an EMT, artificially induced in immortalised mammary epithelial cells, demonstrated stem cell properties and that stem-like cells (CD44<sup>hi</sup>CD24<sup>lo</sup>) isolated from mouse/human mammary glands or carcinomas expressed EMT markers [116]. These findings indicate that epithelial to mesenchymal transitions of differentiated cancer cells may give rise to cancer stem/stem-like cells in breast cancer. However, this study does not address the cell of origin of breast cancer, and in this human cancer at least, the original tumour-initiating cell may be distinct from the cancer stem/stem-like cells involved in tumour progression and metastasis. One study, conducted on circulating tumour cells (CTCs) of metastatic breast cancer patients, demonstrated that a major proportion of these cells were positive for both stem cell and EMT markers, thus providing further evidence for a link between cancer stem cells and epithelial to mesenchymal transition [117].

The cancer stem cell theory holds the cancer stem cell solely responsible for tumour initiation, progression and metastasis. There is much evidence to support the role of EMT in prostate cancer metastasis [115, 118, 119], yet the putative cancer stem cell markers identified by Collins et al. [58] are not entirely consistent with the EMT phenotype therefore, perhaps both have a role to play in prostate cancer. As such, it is plausible that the progeny of transformed stem cells are also tumourigenic and that such progeny may undergo EMT which then confers the ability to invade and metastasise. Therefore, whereas the stem cell may be the initial site of malignant transformation, their transdifferentiating progeny, rather than the cancer(ous) stem cells themselves, may be accountable for invasion and metastasis. As such, the term ‘cancer stem cell’ may be considered to refer to a number of different cell populations including transformed SCs and EMTs, rather than one ‘super-malignant’ cell population, all of which have a role to play in tumourigenesis. Conversely, EMT may be restricted to the cancer stem cell component of a tumour; however, this remains to be demonstrated in prostate cancer. Consequently, in order to achieve full and permanent eradication of the tumour, cancer therapies should aim to target the tumour-initiating cancer stem cells as well as their more differentiated progeny and potentially the populations undergoing EMT.

**Therapies to target prostate cancer stem cells**

Chemotherapy can increase patient survival by a few months, but the quality of life is often reduced by uncomfortable side effects [120, 121], and patients with metastatic disease often fail to respond to treatment. Furthermore, drug-resistant proteins (e.g. MDR1 and ABC transporters) are more likely to be expressed by cancer stem cells than the more differentiated cancer cells and render the CSCs more resistant to chemotherapeutic drugs...
NK cell-activating ligands. Therefore, not only would such expressing cells not express detectable MHC class I or (CD133notherapy. One study, investigating the immunogenicity of developing strategies to target this population using immunomolecules as well as classical HLA molecules, in order to stem cells, with regard to the expression of non-classical it is prudent to investigate the immune status of cancer considering these cells as targets for immunotherapy. Therefore, cancer stem cells would prove problematic when consid-

feron gamma secretion. The expression of HLA-G by (NK) cell-mediated cytolysis and the inhibition of inter-

莓molecules demonstrated the modulation of both adaptive and innate immunity by HLA-G through the suppression of allogenic T-cell proliferation, the expansion of CD4-CD25high-

FOXP3 regulatory T cells, the inhibition of natural killer (NK) cell-mediated cytolysis and the inhibition of interferon gamma secretion. The expression of HLA-G by cancer stem cells would prove problematic when considering these cells as targets for immunotherapy. Therefore, it is prudent to investigate the immune status of cancer stem cells, with regard to the expression of non-classical molecules as well as classical HLA molecules, in order to develop strategies to target this population using immunotherapy. One study, investigating the immunogenicity of putative astrocytoma and glioblastoma cancer stem cells (CD133+ cells), revealed that the majority of CD133-expressing cells did not express detectable MHC class I or NK cell-activating ligands. Therefore, not only would such cells prove resistant to adaptive and innate immune surve-

veillance, they would appear to be unsuitable targets for immunotherapy. However, up-regulation of MHC class I and NK cell ligands was successfully achieved following treatment with interferon gamma, hence the immunogenicity of the cells was restored [128]. Despite low levels of MHC class I being reported in both embryonic stem cells and putative astrocytoma and glioblastoma cancer stem cells, expression of this molecule was dramatically increased in both studies following treatment with interferon gamma. As such, the implementation of interferon gamma in an immunotherapy regimen targeting cancer stem cells may render these cells more susceptible to immune attack.

The MHC status of cancer cells undergoing EMT and the reverse process MET is also of importance when considering these cells as targets for immunotherapy. Furthermore, it is critical to identify unique markers expressed by these cancerous transdifferentiating cells which could be exploited for immunotherapy. Although transcription factors involved in EMT, such as Twist and Snail, may appear attractive targets for cancer therapy, they may not prove to be ideal targets for immunotherapy as EMT is an essential programme required for tissue repair and organ remodelling throughout life. Hence, targeting critical EMT factors may prove detrimental to the patient. It is therefore necessary to identify unique antigens, mutated forms of essential transcription factors/key EMT drivers, or antigens which are only up-regulated in cancer cells undergoing EMT. Epithelial to mesenchymal transition has been reported to play a critical role in cancer progression and metastasis. Providing that cancer cells undergoing these transdifferentiation programmes retain malignant characteristics which distinguish them from healthy cells in both states (epithelial and mesenchymal), these cells may prove to be ideal targets for immunotherapy.

Comparison of gene-expression profiles of normal tissues/stem cells versus cancer cells/cancer stem cells can identify novel target antigens for therapy which are preferentially expressed in cancer stem cells [122]. Patients who present a macroscopic tumour could be given immuno-

therapy against the antigens found on the more differentiated cells in order to remove the bulk of the tumour, as a first-line treatment, while patients who present residual disease following a regimen of therapies could be given immunotherapy tailored against the cancer stem cell-spe-

cific antigens [129]. Yawata and colleagues have recently demonstrated the transcriptional activation of the cancer testis antigen (CTA) gene locus in glioma stem cells which showed frequent and high expression of CTA genes [130].

Most of the researchers working on the subject of prostate cancer stem cells agree that studies must be carried out on primary cancer cells rather than cancer cell lines,
whenever possible. Changes in the environment are inevitable in in vitro cell culture and in vivo animal models/xenografts, and these could be inducing a deregulation of the expression of cancer stem cell markers leading to inaccurate measurements of their activity. It is therefore essential to precisely recreate the tumour stroma/microenvironment to allow observation and report of true phenomena in order to develop effective therapeutics [131, 132].

Concluding remarks

Although the clinical responses observed with the current immunotherapies are encouraging, they are not entirely satisfactory [133, 134]. For instance, an important drop in immunotherapies are encouraging, they are not entirely essential to precisely recreate the tumour stroma/microenvironment to allow observation and report of true phenomena in order to develop effective therapeutics [131, 132].

References


