Control of gene expression in prostate epithelial cell differentiation hierarchy

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ABSTRACT

The aim of present investigation is to elucidate the complex stem cell (SC) dynamics within prostate cancer, which can be exploited to design novel diagnostic and therapeutic strategies for the management of prostate cancer. In order to determine the precise transcriptional and microRNA regulatory mechanisms modulating SC self-renewal and differentiation, unique cellular assays have been developed in our lab. These assays utilise homogeneous cell sub-populations enriched from patient-derived prostate cultures. Occasionally, cell line models and patient-derived mouse prostate cancer xenografts were also employed.

Using a prospective bioinformatic analysis of gene expression data from Birnie et. al., 2008, we have identified LCN2, CEACAM6, and S100p as candidate genes for regulation of prostate SC differentiation. These genes are over-expressed in differentiated cells, compared to SC, and have a more similar expression pattern with each other than with any other gene. Since their promoters have binding sites for 32 common transcription factors, the genes may therefore form a co-regulated network and/or have similar functions. Retinoic acid treatment can also induce the expression of all these genes, suggesting that LCN2, CEACAM6, and S100p may play an important role in retinoic acid-mediated prostate epithelial SC differentiation. The genes could also be so-regulated by miR-128, miR-188, and miR-548c, based on an analysis of the miRNA expression by microarray generated in this work. Patient-derived prostate epithelial sub-populations enriched from PrEC, BPH, PCa, and CRPC were profiled for the expression of 766 miRNAs. This analysis identified a very specific prostate cancer SC miRNA signature, and showed that miRNA expression can distinguish between PCa and CRPC. The integration of this miRNA microarray data with gene expression microarray data showed that pathways regulating both the cell cycle (SC quiescence) and cell-cell interaction (SC-stromal niche interaction) could be significantly influenced by miRNAs during differentiation. A lack of telomerase expression/activity in prostate cancer SCs, in contrast to their differentiated progeny also points towards the quiescent nature of these cells. The telomerase studies further revealed that BPH is a disease sustained by progenitor proliferation and that inhibition of telomerase in BPH derived SCs can suppress their self-renewal; while cancer SC self-renewal is not affected by telomerase inhibition.

We anticipate that these results, with further functional studies, will comprehensively establish a detailed knowledge base for regulatory mechanisms active in prostate

SC and prostate cancer SC differentiation. This data will be invaluable in formulating efficient management strategies for prostate cancer.

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AUTHOR'S DECLARATION

I declare that this thesis represents my own unaided work, except where acknowledged otherwise in the text, and has not been submitted previously in consideration for a degree at this, or at any other university.

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- **Rane JK**, Droop AP, Pellacani D, Polson EP, Simms MS, Mann VM,, Maitland NJ. Retinoic acid controlled gene-network regulates early stages of prostate epithelial stem cell differentiation during benign and malignant development.

- **Rane JK**, Pellacani D, Maitland NJ. Does telomerase represent a valid target for prostate cancer management?

- **Rane JK**, Pellacani D, Maitland NJ. The emerging role of miRNAs in the regulation of prostate cancer stem cell phenotype

‘….. in science and all its applications, what is crucial is not that technical ability, but it is imagination in all of its applications. The ability to form concepts with images of entities and processes pictured by intuition’

--E. O. Wilson, American biologist, researcher, theorist, naturalist and author.

‘Our whole theory of education is based on the absurd notion that we must learn to swim on land before tackling the water’

--Henry Miller, American writer and painter.

1. INTRODUCTION

The human prostate is a small walnut sized exocrine gland of the male genitourinary system. It is located at the neck of urinary bladder surrounding the urethra. In post-pubertal males, the prostate secretes an acidic fluid containing proteins and zinc, which ensure optimal sperm functionality in the female genital tract (Aumuller, 1983). Although prostate dysfunction rarely contributes to infertility, with advancing age it can suffer from prostatitis, benign prostatic hyperplasia (BPH), and prostate cancer. These disorders are very common; for example, the incidence of BPH by the age of 40 years is about 23% of the total population, and it increases roughly by 10% every decade thereafter (Cunha et al., 1987). Prostate cancer is the commonest cancer among males in the UK (Cancer Research UK report, 2012). Therefore, a better understanding of prostate homeostasis is essential to improve the management of these disorders.

1.1 The anatomy of the prostate:

The human prostate can be divided into four zones (**Figure 1.1**). Each of these zones has a specific location and a variable content of exocrine acini and fibro-muscular stroma (McNeal, 1981, Fine and Reuter, 2012).

- The peripheral zone (PZ) is the largest zone of the prostate, containing about 70% of the glandular prostate. It surrounds the urethra and is the most common site of origin of prostate cancer.
- The central zone (CZ) is the second largest zone. It contains the ejaculatory ducts – about 20% of prostate cancers arise from this zone.
- The transitional zone (TZ) also surrounds the urethra and is the exclusive site of BPH origin.
- The anterior zone (AZ) is a fibro-muscular zone, which lacks glands.

This anatomical arrangement is remarkably different from the rodent prostate, which is the commonest animal model for investigations of prostate physiology and pathology. In rodents, the prostate does not have zones, but very distinct lobes (Marker et al., 2003).

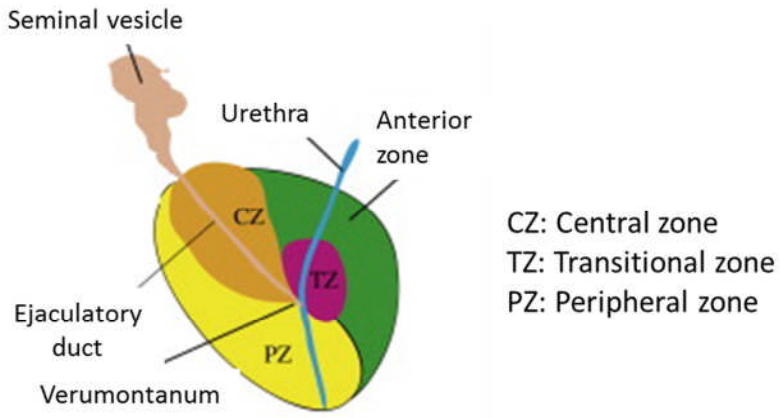


Figure 1.1: Schematic diagram showing zonal architecture of human prostate, sagittal view (Zhai et al., 2010).

1.2 Prostate histology:

The human prostate has a high level of organisation. It has bilayered epithelial acini and fibro-muscular stroma, separated from each other by a basement membrane (**Figure 1.2**). Three phenotypically distinct cell types can be identified within the epithelial bilayer: the basal cell, the neuroendocrine cell, and the luminal cell.

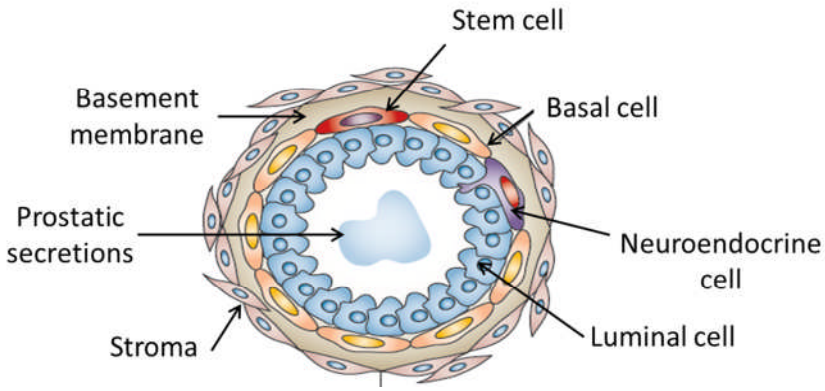


Figure 1.2: Schematic representation of the prostrate acinus. Adapted from (Rane et al., 2012).

- Basal cells: These cells form a relatively proliferative, androgen-independent but androgen responsive cell layer (English et al., 1987, Kyprianou et al., 1996, van Leenders et al., 2000). Basal cells are in a direct contact with the basement membrane, which provides a structural barrier between the epithelium and the stroma. The majority of investigations show that the basal cells are the non-secretory precursors of the luminal cells in adult prostate (Isaacs and Coffey, 1989, Robinson et al., 1998, Hudson et al., 2000, Richardson et al., 2004, Frame et al., 2010, Ousset et al., 2012), however, the contradictory evidence also exists (Wang et al., 2009, Choi et al., 2012). Basal cells can be characterised by the expression of P63 (Signoretti et al., 2000), CD44 (Liu et al., 1997), c-MET (van Leenders et al., 2003), oestrogen receptor β (Aumuller, 1983), and cytokeratin 5/14 (Verhagen et al., 1992). This cell layer also displays variable expression of mitosis suppressor $p27^{Kip1}$ expression (7-10%) (De Marzo et al., 1998). The prospective prostate epithelial stem cells are suggested to reside in the basal layer of the human prostate (Richardson et al., 2004, Goldstein et al., 2008, Garraway et al., 2010) and in the rodent prostate (Burger et al., 2005, Lawson et al., 2007, Goldstein et al., 2008, Leong et al., 2008, Burger et al., 2009).
- Neuroendocrine cells: These rare cells are located in the basal epithelial layer. These androgen insensitive cells secrete peptide hormones and biogenic amines, such as chromogranin-A, serotonin, bombesin, calcitonin, and parathyroid hormone-related peptide (Abrahamsson and di Sant'Agnese, 1993, Nakada et al., 1993, Rumpold et al., 2002). The precise origin and the function of these cells in the prostate, is however still unclear.
- Luminal cells: These are the terminally differentiated columnar cells that are responsible for producing prostatic secretions. These secretions are composed of enzymes and metal elements, such as prostatic acid phosphatase (PAP), prostate specific antigen (PSA) and zinc. Luminal cells

have no proliferative potential and are characterised by the near universal expression of androgen receptor (AR) (Nakada et al., 1993, Wang et al., 2006a), NKX3.1 (Wang et al., 2009), CD57 (Signoretti et al., 2000), CD24 (Liu and True, 2002, Lawson et al., 2007), and cytokeratin 8/18 (Verhagen et al., 1992, Robinson et al., 1998, van Leenders et al., 2000). Almost all of the luminal cells (85-100%) express a marker for non-proliferating cells $p27^{Kip1}$ and a correspondingly negative expression of proliferation markers PCNA and Ki-67 (De Marzo et al., 1998).

1. 3 Disorders of the prostate:

The prostate is a common site of inflammatory and tumorigenic lesions. There are three distinct prostate pathologies: prostatitis, benign prostatic hyperplasia, and prostate cancer.

1.3.1 Prostatitis:

This is the most common disorder of the prostate. Prostatitis (predominantly inflammation of the central zone) presents as an acute or chronic (most common presentation) lower abdominal pain with increased urinary frequency, or it could also remain asymptomatic (Sharp et al., 2010). The retrograde transmission of infectious agents (less often) and damage caused by passing urinary toxins, stress, autoimmunity, and physical trauma in the central zone are some of the most common aetiological factors for prostatitis (Krieger, 2004, Sharp et al., 2010). Chronic prostatitis is widely considered as a risk factor for the development of BPH and even prostate cancer (Lee and Peehl, 2004, Kramer and Marberger, 2006, Kramer et al., 2007, Nickel, 2008, Sciarra et al., 2008). Recent semen analysis studies have found that about 20-30% of young healthy men (18-25 years of age) have some degree of stroma-driven asymptomatic inflammatory prostatitis, suggesting that there could be a prolonged inflammatory insult to the prostate

epithelium (Carver et al., 2003, Korrovits et al., 2008). This could lead to repeated attrition of the luminal layer, prompting reactionary hyperplasia.

1.3.2 Benign prostatic hyperplasia (BPH):

BPH is a non-malignant nodular hyperplasia of both the stroma and the epithelium of the prostate, almost exclusively arising from periurethral transitional zone (Nickel and Roehrborn, 2008). Age-related deregulation in a balance between androgenic and peptide growth factor signalling (e.g. IGF-1) is considered as the prime aetiological factor for the development of BPH (Thorpe and Neal, 2003, Roehrborn and McConnell, 2007). It is often a slow growing lesion and usually presents after the age of 50 years, with symptoms such as, dribbling at the end of urinating, urinary hesitancy, dysuria, and urinary retention (Roehrborn, 2011). The severity of symptoms and personal preference of patients determine the treatment choice (Thorpe and Neal, 2003, Roehrborn, 2011). For mild symptoms, watchful waiting along with 5-α-reductase inhibitors is usually recommended. Minimally invasive operations, such as laser prostatectomy are available for patients with more severe symptoms. However, the commonest treatment is trans-urethral resection of prostate (TURP) (Roehrborn, 2011). TURP cures BPH patients in about 95% of the cases (Varkarakis et al., 2004). The remaining patients need a second operative procedure. It is widely accepted that BPH is not a precancerous lesion, although it increases the coincidental diagnosis of incipient prostate cancer (Orsted et al., 2011, Chang et al., 2012).

1.3.3 Prostate cancer:

Prostate cancer accounts for every fourth patient diagnosed with cancer in the UK (Cancer Research UK report, 2012). The vast majority of these cancers are adenocarcinomas (99%), with minimal presence of ductal carcinoma (0.141%), mucinous adenocarcinoma (0.103%), small cell carcinoma (0.056%),

carcinosarcoma (0.07%) and embryonal carcinosarcoma (0.06%) variants (Kendal and Mai, 2010). Therefore henceforth, prostate cancer will be discussed only in relation to the prostate adenocarcinoma.

1.3.3.1 Aetiology and risk factors:

- **Age:** Advancing age is by far the most well-established risk factor for the development of prostate cancer (Crawford, 2003). The incidence of prostate cancer increases with advancing age, and at the age of 90 almost all males have at least histological evidence of prostate cancer (Cancer Research UK report, 2012). However, the differences in the age at diagnosis, heterogeneity in geographical incidence, histology, progression, and treatment response suggest that there are additional contributory risk factors.
- **Ethnicity:** The incidence and disease aggressiveness is seen more in the patients of African ancestry compared to white Americans/Europeans and the Hispanics and those of Asian origin, who appear to be less susceptible to the development of prostate cancer (Brawley, 2012).
- **Inflammation:** There is no conclusive data, but compelling evidence is accumulating for the co-relation between the presence of chronic inflammation, proliferative inflammatory atrophy of the prostate, and mutations or epigenetic alterations in the genes important in the inflammatory pathways (e.g. RNASEL, MSR1, and GSTP1) with the subsequent/concurrent diagnosis of prostate cancer (Nelson et al., 2004, Goldstraw et al., 2007). Inflammation inducing chemical agents, such as the charred meat carcinogen PhIP (2-amino-1methyl-6phenylimidazo-[4,5-b]-pyridine), are strongly implicated in the initiation and progression of prostate cancer (Shirai et al., 1997, Tang et al., 2007). PhIP is even being used to generate pre-neoplastic prostate cancer lesion (prostatic intraepithelial neoplasia-PIN) in rodent models (Borowsky et al., 2006).

- **Environmental and dietary factors:** The relationship between most of the environmental and dietary factors is associative and there is insufficient data to confirm any causal relationship. An excessive intake of red meat (Joshi et al., 2012), high fat diet (Kristal et al., 2010), and calcium is proposed to increase the risk of having high grade prostate cancer; whereas, intake of food with vitamin-like molecules lycopenes (present in tomatoes, watermelon etc.) could be protective against prostate cancer. Multiple clinical trials evaluating the role of dietary supplement with food containing lycopenes and other vitamins (beta-carotene, folates, retinol and vitamin E) have however failed to identify significant prostate cancer preventive effects (Beilby et al., 2010, Kristal et al., 2011).
- **Genetic and epigenetic changes:** Causal hereditary genetic and epigenetic alterations are relatively uncommon in prostate cancer. Familial predisposition for prostate cancer is seen in only about 10% of the total cases (Stanford and Ostrander, 2001). In familial prostate cancer, 3 successive generations or 2 siblings have diagnosed prostate cancer at the age <55 years. The rest of cases demonstrate some recurrent genomic alterations in addition to genetic and epigenetic abnormalities (**Table 1.1** and **Figure 1.3**). It has been proposed that about 42% of the risk of prostate cancer can be attributed to genetic influences (Hsing and Chokkalingam, 2006). Changes in the expression of genes with some other less frequent abnormalities interfere with several key signalling pathways, such as, mTOR/AKT pathway, NF-κB signalling pathway, MAPK pathway, the EZH2 mediated signalling pathway, and developmental signalling pathways (Shen and Abate-Shen, 2010). However, a direct causal role of these anomalies 'in isolation' has not been conclusively demonstrated. Recent studies have indicated that miRNA deregulation could also be critical for the pathogenesis of prostate cancer (Please refer to SECTION III).

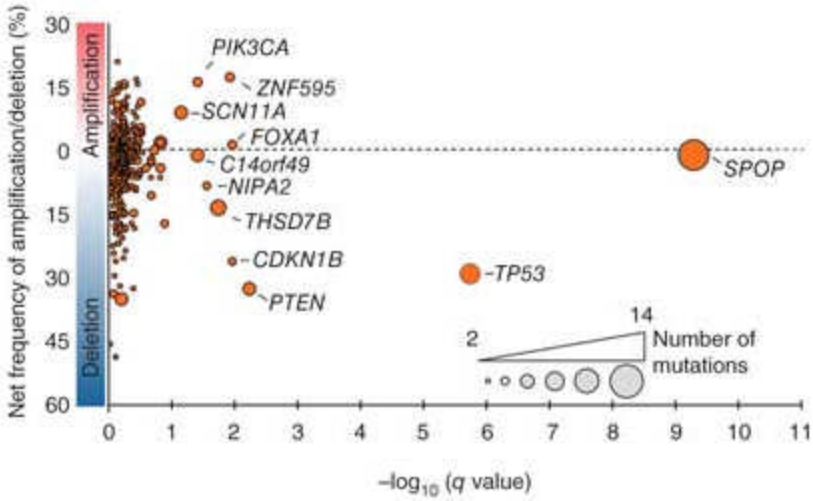


Figure 1.3: **Common genetic alterations in prostate cancer.** Net frequency of autosomal genes with two or more mutations deletion and/or amplification in prostate cancer. Significantly mutated genes are indicated (Barbieri et al., 2012).

Gene	Chromosome	Event	Function
NKX3.1	8p	Inactivation	Cell growth/differentiation
c-MYC	8q	Genomic amplification	Cell growth/differentiation
PTEN	10q	Inactivation	Cell growth/differentiation
GSTP1	11q	Promoter methylation	Oxidative stress response
RB	13q	LOH, mutation	Cell cycle regulation
P53	17p	LOH, Mutation	Cell cycle regulation, apoptosis, DNA damage repair
ETS genes	21q	Fusion with AR targeted genes (TMPRSS2)	Cell growth/differentiation
AR	Xq	Mutation, genomic amplification	Cell growth/differentiation

Table 1.1: Common genetic and epigenetic alterations found in the prostate cancer. Adapted from (Koochekpour, 2011).

- **Androgen signalling:** Paracrine androgen signalling mediated by stroma is essential for the development and maintenance of the prostate epithelium (Kurita et al., 2001, Gao et al., 2006). Both the context and cell-type dependent gain and loss of function in AR expression are implicated in the progression of

prostate cancer (Shen and Abate-Shen, 2010). However, recent reports in rodent and human cell culture models indicate that prostate cancer stem cells could be AR-responsive, but AR independent (Collins et al., 2005, Goldstein et al., 2010, Lawson et al., 2010, Qin et al., 2012). This suggests that AR signalling could be essential for cell proliferation leading to increased tumour mass, but may not be necessary for tumour initiation.

1.3.3.2 Clinical presentation - symptoms and signs:

With routine PSA screening (in USA) and increased awareness, the majority of cases of prostate cancer is diagnosed even before the development of urinary tract symptoms (Miller et al., 2003). In other cases, it may present with increased urinary frequency, hematuria, and decreased urine stream (Fitzpatrick et al., 1998, Chen, 2001). Patients with advanced and metastatic disease may present with weight loss, anaemia, and bone pain (Chen, 2001, Heidenreich et al., 2011). The symptoms are associated with signs, such as bone marrow suppression, a hard mass on digital rectal examination, and high PSA.

1.3.3.3 Diagnosis:

The widespread incidence and relative ease in treatment of localised disease has prompted a call for routine serum prostate specific antigen (PSA) screening. PSA is a serine protease, which maintains the fluidity of seminal fluid (Lilja, 1985). An increase in serum PSA is considered as an indicator of increasing prostate gland volume and prostate cancer progression (Hudson et al., 1989). Routine serum PSA screening is however performed in very few countries (e.g. the USA and Austria), but most European countries do not recommend it (Heidenreich et al., 2011). The principal reasons for this hesitancy in European countries could be due to: (i) high levels of false positivity with PSA testing and (ii) the inability of PSA to distinguish between aggressive and indolent cancers (Schroder et al., 2009, Andriole et al., 2012). The utilisation of newer, more specific and sensitive biomarkers, such as

PCA3 and circulating tumour cells, could be a more promising option for the screening and risk stratification of prostate cancer (de Kok et al., 2002, Hessels et al., 2003, Danila et al., 2007). However, determination of serum PSA levels is still vital for the diagnosis and the evaluation of treatment response.

The main tools to diagnose prostate cancer include digital rectal examination, serum PSA concentration, and transrectal ultrasound-guided biopsies (Heidenreich et al., 2011). Conclusive diagnosis of prostate cancer can only be established after histological examination of biopsies (van der Kwast et al., 2003). The histological features are graded according to the Gleason grading (Gleason, 1966, Gleason, 1992). Prostate cancer can be graded from 1 to 5, based on the histological architecture of the prostate gland and differentiation status of the tumour (grade 1 being closer to the normal histology and 5 being the most disrupted archetecture) (**Figure 1.4**). For the final report, the score of the two most prevalent grades is added together to give a Gleason score between two and ten. For example, for Gleason score 7 could be (4+3) or (3+4). In the 4+3 case, the most prevalent grade is 4 and the second most prevalent grade is 3. In 3+4 case, the most prevalent grade is 3 and the second most prevalent grade is 4. According to the system adopted in 2005, the following information should also be mentioned to aid treatment choice: (i) for each biopsy site, the proportion of biopsies positive for carcinoma, (ii) the proportion (percentage) or length (in millimetres) of tumour involvement per biopsy, (iii) the presence of high-grade PIN and perineural invasion (Epstein et al., 2005). About half of the patients are diagnosed at a very early stage of the disease, with Gleason grade ≤ 6 (Brawley, 2012). Tumour TNM staging is also necessary, before deciding on the appropriate treatment, where T(0-4): Tumour localisation with respect to the prostate gland, N(0/1): lymph node status, and M(0/1): presence of distant metastasis (Schroder et al., 1992). The presentation pattern of cancers in UK is described in **Table 1.2**.

Figure 1.4: Schematic representation of the Gleason grading of prostate cancer (Gleason, 1966, Gleason, 1992).

A

Gleason grade at diagnosis	% of cases
< 6	~2%
6-7	~75%
8-10	~23%

B

Extent of tumour spread at diagnosis	% of cases
Organ confined cancer	~70%
Local and systemic spread	~30%

Table 1.2: The extent of prostate cancer growth and aggressiveness at diagnosis in the UK (The British Association of Urological Surgeons, 2010). A: Gleason grade at diagnosis B: Extent of tumour growth at diagnosis.

1.3.3.4 Treatment:

Any decision for therapy depends upon the stage of the disease, histological grade of the tumour and the patient choice. The options available are described in **Table 1.3**.

Treatment option	Indications	Comment
Active surveillance	• Clinically confined PCa (T1–T2) • Gleason score ≤ 6 • Three or fewer biopsies involved with cancer • ≤ 50% of each biopsy involved with cancer • PSA < 10 ng/ml	Annual surveillance re-biopsies are recommended in addition to PSA testing
Radical prostatectomy (RP)	• Low- and intermediate-risk localised PCa (cT1a–T2b) • Gleason score 2–7 • PSA ≤ 20 ng/ml • A life expectancy >10 yr	The prostate gland is removed. Lymph nodes near the prostate may be removed.
Radiotherapy	• Patients who refuse surgical intervention • An option for patients with cT1–T2a, Gleason score <7, PSA ≤ 10 ng/ml, prostate volume ≤ 50 ml, without a previous TURP • Immediate postoperative external irradiation after RP for patients with pathologic tumour stage T3 N0 M0 • Immediate postoperative external irradiation after RP for patients with positive surgical margins • Concurrent admission with hormonal therapy for metastatic disease	Intensity modulated radiotherapy (IMRT), an optimised form of three dimensional conformal radiotherapy (3D-CRT) is the gold standard
Androgen deprivation therapy (ADT) and chemotherapy	• Advanced metastatic prostate cancer • Recurrent prostate cancer	• ADT: Anti-androgens (e.g. Bicalutamide), LHRH-agonist (e.g. Zoladex), Oestrogen treatment (e.g. Stilbestrol), GnRH-antagonist (e.g. Finasteride). • Chemotherapeutic drugs (e.g. Docetaxel)

Table 1.3: Treatment options for prostate cancer management. Compiled from (Heidenreich et al., 2011, Schroder et al., 2012). PCa: prostate cancer, TURP: transurethral resection of prostate, PSA: prostate specific antigen, RP: radical prostatectomy, LHRH: Luteinising hormone releasing hormone, GnRH: Gonadotropin releasing hormone.

In addition to the treatment options mentioned in the table, cryosurgical ablation of the prostate (CSAP) (Babaian et al., 2008) and high-intensity focussed ultrasound (HIFU) (Warmuth et al., 2010) have emerged as alternative therapeutic options in patients with clinically localised prostate cancer. A regular follow-up is necessary after any of these treatments. Disease progression can be monitored by regular PSA testing and digital rectal examination with CT/MRI scans (if necessary) to detect systemic spread (Heidenreich et al., 2011). However, about 20% of advanced cancers fail to respond to androgen deprivation therapy, known as castration resistant prostate cancers (CRPCs). It is unclear why such an androgen dependent tumour fails to respond to androgen deprivation. One of the hypotheses is that androgen deprivation leads to the accumulation of androgen independent stem and basal cells (Shen and Abate-Shen, 2010, Rane et al., 2012). These cells can then drive further tumour progression. The pathophysiology and treatment options are discussed in the 'genetic regulation of prostate stem cell differentiation' introduction section (SECTION I).

The development of CRPC, over-representation of basal cells in the CRPCs, and the analysis of normal prostate epithelium have suggested that the prostate epithelium is arranged into a hierarchy, which is composed of relatively functionally distinct cell sub-populations (Maitland and Collins, 2008b). Selection of pure and homogeneous cell sub-populations would help to explore only the fundamental causes of heterogeneity, minimising the noise created by differences in cellular differentiation and the variable cellular composition of the tumour mass (differential content of luminal, basal, endothelial, stromal, and inflammatory cells).

1.4 Adult stem cells and cellular hierarchy of adult tissues:

Similar to the development of an entire organism from a single fertilised egg, organs such as prostate are believed to be derived from stem cell driven cellular hierarchy (Isaacs and Coffey, 1989). This cellular hierarchy can be described as a pyramid of functionally heterogeneous cells, in which a rare stem cell population residing at the apex generates all the differentiated populations (**Table 1.4**). The categorisation of cells is based upon spatial and functional foundations, where stem cells are defined as long-living cells that reside at particular micro-environmental niche and possess the ability to self-renew, proliferate and differentiate into all the cell lineages that constitute the tissue (van der Kooy and Weiss, 2000) (**Table 1.4**). Several lines of evidence stress the importance of a stem cell niche, e.g. niche disruption leads to haematopoietic stem cell differentiation (Kirstetter et al., 2006) and an ageing niche affects muscle stem cell functionality, even in vivo (Chakkalakal et al., 2012). As cell lines grown in 2-D culture for a prolonged time lack tissue homeostasis, spatial localisation of cells (niche), and are under continuous selection pressure mainly on the basis of proliferative potential (not the principal selection criteria in tissue); a cell line model is not likely to fulfil the functional criteria for the establishment of a hierarchy. Long-term culture of cells in serum-containing media can also induce chromosomal changes (Lee et al., 2006, Izadpanah et al., 2008) and DNA hypermethylation (Antequera et al., 1990). Therefore, stem cell related data exclusively or heavily relying only on cell line models are not discussed here.

Stem cell attribute	Comment
Rarity	Exceptions are noted (e.g. Melanoma)
Pluripotency	Can differentiate into all the cell lineages identifiable in the tissue
Quiescence	Slowly cycling cells but have a large proliferative potential
Self-renewal	Can divide asymmetrically to form one differentiated and another stem cell
Long living cells	Their ability to self-renew and remain quiescent enables stem cell to provide cell reserves that exceeds the lifetime of the individual
Specific location in the niche	Attachment with the basement membrane and contact with the niche cells is essential to maintain above mentioned characteristics

Table 1.4: Characteristics of stem cells. Adapted from (Miller et al., 2005).

The concept of stem cells gained its first major supportive evidence in early 1960's, when pioneering work of Till and McCulloch with spleen colony forming assays demonstrated for the first time that a rare population of cells exists in mouse bone marrow that satisfy the above mentioned stem cell criteria (Till and Mc, 1961, Becker et al., 1963). In next 3 decades, stem cells were isolated and characterised from several adult animal and human organs, such as haematopoietic system (Spangrude et al., 1988, Bernstein et al., 1994), brain (Temple, 1989, Reynolds and Weiss, 1992), skin (Cotsarelis et al., 1990, Lyle et al., 1998), and breast (Kordon and Smith, 1998, Welm et al., 2002, Shackleton et al., 2006). In late 1980's, based on classical serial prostate involution-regeneration experiment in rats, the existence of prostate epithelial stem cells was also proposed, indicating a hierarchical arrangement within the prostate epithelium (English et al., 1987, Isaacs and Coffey, 1989).

1.4.1 Hierarchy in normal prostate epithelium

Ever-improving molecular and cellular techniques are providing enticing glimpses of the structural and functional architecture of the prostate epithelium. Over the last few years, series of experiments performed in rodents has established beyond reasonable doubt that a self-renewing population of stem cells exists in the basal prostate epithelial layer. The initial indications came from cyclical ablation-regeneration of the rat ventral prostate (English et al., 1987, Kyprianou and Isaacs, 1988, Walensky et al., 1993) (**Figure 1.5**). These experiments showed that the prostate gland can regenerate itself even after prolonged androgen ablation. Two experiments conducted during the last decade, further showed that transplantation of even a single clonogenic prostate epithelial cell with embryonic mesenchyme could regenerate an entire prostate in mice (Xin et al., 2003, Azuma et al., 2005). Additional experiments have also identified long-term BrdU-label retaining infrequent cells in the basal epithelial layer of the proximal duct region (Tsujimura et al., 2002). Taken together, these evidences suggested the presence of a population of long surviving cells with stem cell characteristics in the basal prostate epithelial layer of the proximal duct region of a rodent prostate. Subsequently, cells that were clonogenic and could reconstitute prostate were precisely isolated by enriching basal epithelial cells for SCA-1 (Burger et al., 2005), TROP2 (Goldstein et al., 2008), and ALDH (Burger et al., 2009) cell surface molecules. Further experiments demonstrating distinct lineage differentiation of rare mouse prostatic basal epithelial cells with an expression profile $Lin^{-}Sca\text{-}1^{+}CD49f^{hi}$ or $Lin^{-}Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$, is clearly strengthening the prostate stem cell hypothesis (Lawson et al., 2007, Leong et al., 2008). However, Wang and colleagues recently identified a second distinct population of stem cells in the mouse luminal cell layers (Wang et al., 2009). They showed that luminal castration-resistant NKX3.1 positive cells exhibited self-renewal and *in-vivo* prostate reconstruction ability at a single cell

level. Two recent lineage-tracking studies marked the K5/14/8 expressing cells and found that both basal and luminal progenitor cells exist in mouse prostate epithelium, with some additional evidence for basal cell differentiation into luminal cells (Choi et al., 2012, Ousset et al., 2012). Even though there is no consensus on the location and number of stem cells in the murine prostate, these testable models have immensely contributed to fine-tune concepts and techniques that can be applied for the investigations of human prostate epithelium.

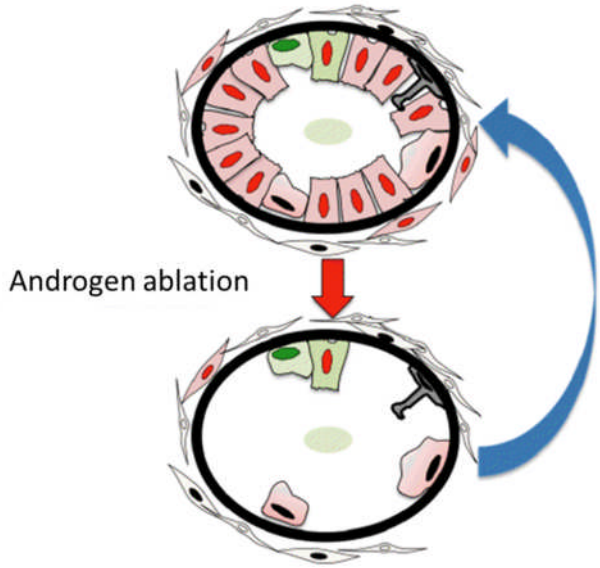


Figure 1.5: Schematic representation of serial androgen ablation and regeneration of a rodent prostate. Adapted from (Maitland, 2012, Rane et al., 2012)

The identification of human prostate epithelial stem cells was however seriously hampered due to technical challenges involved in maintaining human prostate epithelial cultures and isolating pure and homogeneous cell sub-populations. On the basis of immunoprofiling with Ki-67, PCNA, and MIB1, initial experiments showed that the proliferative compartment of normal prostate lies in the basal epithelial layer (Bonkhoff et al., 1994). However, only 1 in 200 of basal cells were found to generate compact colonies reminiscent of colonies derived from stem cells in two-dimensional culture and structures similar to normal prostate epithelium in three-

dimensional matrigel culture (Hudson et al., 2000). Later, Hoechst 33342 exclusion flow-cytometry experiments suggested that a putative rare stem cell population, which is predominantly basal and quiescent, could be enriched from the tail region of a side population (Bhatt et al., 2003). These experiments implied the existence of stem cells in the basal layer of the human prostate epithelium, but the surface marker and molecular profile of prostate stem cells still remained elusive. A basal cell fraction enriched for $\alpha_2\beta_1$-integrin and CD133 was then shown to exhibit high proliferative capacity under appropriate culture conditions and an ability to reconstitute prostate glands *in vivo* (Collins et al., 2001, Richardson et al., 2004). An obvious morphogenesis and epithelial cytodifferentiation was noted when $\alpha_2\beta_1$-integrin^{hi}/CD133^{+} cells were transplanted with prostate stroma in athymic nude mice, with some acini having well-defined lumen and distinct basal and luminal layers (Richardson et al., 2004). Subsequent identification of a sphere forming basal cell sub-population enriched for TROP2 and CD49f that can self-renew and had differentiation potential *in vivo* again pointed towards the existence of basal stem cell population in human prostate (Goldstein et al., 2008, Garraway et al., 2010). Therefore, now it has been widely accepted that prostate stem cells reside in the basal epithelial layer and drive a dynamic human prostate epithelial hierarchy.

Distinct basal and luminal layers can be distinguished on the basis of morphology, differential cytokeratin and surface marker expression, and expression of some cell specific proteins such as P63 for basal cells and PSA for luminal cells (McDonnell et al., 1992, Liu et al., 1997, Signoretti et al., 2000, Patrawala et al., 2006). The human prostate stem cell resides in the basal epithelial layer, close to the basement membrane and can differentiate into committed basal cells, which in turn terminally differentiate into luminal cells (Robinson et al., 1998, Collins et al., 2001, Goldstein et al., 2008, Maitland and Collins, 2008a). However, differentiation from stem cells to basal cells is a gradual process and a noticeable intermediary transit amplifying

cell population can be identified in mouse and human prostate epithelia (Isaacs and Coffey, 1989, Hudson et al., 2000, Richardson et al., 2004) (**Figure 1.6**). These cells cannot self-renew, but can proliferate significantly and express basal cell markers. On differentiation into committed basal cells, the transit-amplifying cells lose their significant proliferation potential. When these basal cells move down the hierarchy by terminally differentiating into the luminal cells, they move away from the basement membrane into the luminal layer. The cells with intermediate basal and luminal phenotype beautifully capture this transition on electron microscopy (Brandes, 1966). These intermediate cells predominantly reside in the basal layer, but express some of the classical luminal markers, such as AR (Nakada et al., 1993, Wang et al., 2006a), NKX3.1 (Wang et al., 2009), and cytokeratin 8/18 (Verhagen et al., 1992, Robinson et al., 1998, van Leenders et al., 2000). Furthermore, recent lentivirus lineage tracking experiments clearly demonstrated the ability of human prostate basal cells to differentiate into luminal cells (Frame et al., 2010). Taken together, these findings suggest that the prostate epithelium has a hierarchical architecture. Interestingly, along with normal prostate epithelium, prostate cancer has also been suggested to exhibit cellular hierarchy.

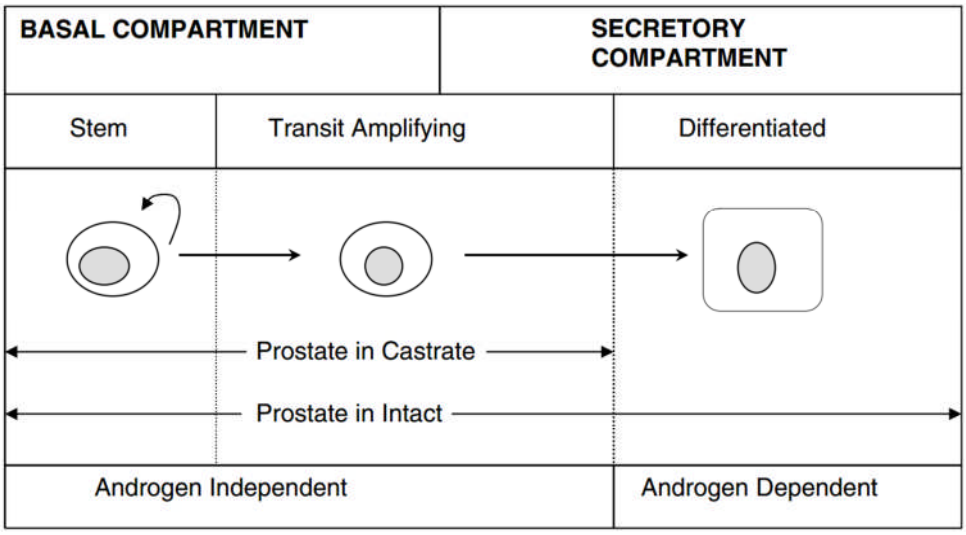


Figure 1.6: Hierarchy in the normal prostate epithelium (Maitland et al., 2006).

1.5 Hierarchy in prostate cancer

Only a rare population of 'cancer stem cells' (CSCs) has been proposed to possess the ability to self-renew, differentiate into heterogeneous cell lineages, and to reconstitute the tumour in vivo (Maitland and Collins, 2005, Clarke et al., 2006). Even though the existence of these CSCs in cancer was proposed in the nineteenth century by Rudolf Virchow, CSCs were only recently identified and characterised in multiple human malignancies, such as leukaemia (Bonnet and Dick, 1997), and cancer of brain (Singh et al., 2003), breast (Al-Hajj et al., 2003), and colon (O'Brien et al., 2007). In the prostate, the propagation and prolonged serial passaging of primary and metastatic prostate cancer tissue in immune deficient mice gave the first indication that prostate cancer may also harbour a CSC population (Wainstein et al., 1994, Pinthus et al., 2000, Corey et al., 2003). Subsequently, a pure and homogeneous putative CSC population with a basal cell phenotype that have proliferative capacity *in vitro,* and the ability to differentiate *in vivo* was successfully enriched using cell surface profile $CD44^{+}/\alpha_2\beta_1 integrin^{hi}/CD133^{+}$ from primary human prostate cancer samples (Collins et al., 2005). CSC-like cells were also successfully characterised from human xenograft tumours using CD44 and $\alpha_2\beta_1$integrin (Patrawala et al., 2006, Patrawala et al., 2007) and TRA-1-60, CD151 and CD16 (Rajasekhar et al., 2011) as markers. Although there is no clear consensus so far, the cell of origin of these CSCs is considered to be within the basal epithelial layer (Goldstein et al., 2010, Lawson et al., 2010, Maitland et al., 2011). Moreover, the general opinion is tending more towards the hypothesis that the human prostate cancer also has a hierarchical structure similar to normal prostate epithelium and it is driven by CSCs.

Multiple studies point out that CSCs possess certain advantageous traits such as quiescence, residence in a specialised niche, special DNA repair mechanisms, and immune modulation. These properties are absent in their differentiated progeny. As

a consequence, CSCs can mount a successful barrier against conventional anti-cancer drugs to which, their differentiated progeny is susceptible (Zhou et al., 2009a, Alison et al., 2011). Treatment of cancer with conventional anti-cancer drugs could therefore result in selective loss of differentiated cells, leading to a response similar to wound healing, causing rebound increase in number of CSCs. The phenomenon of enrichment of CSCs after chemotherapeutic treatment has been indeed observed in cancers of breast (Gupta et al., 2009, Hoey et al., 2009), pancreas (Mueller et al., 2009), liver (Ma et al., 2008), lung (Levina et al., 2008), and colon (Dylla et al., 2008). These CSCs then underpin subsequent development of more aggressive cancer phenotype. Therefore, it has become evident that a combination of drugs targeting CSCs with conventional anti-neoplastic treatments is necessary for efficient cancer management.

In order to investigate the prostate hierarchy, and the mechanisms responsible for prostate cancer progression, multiple models such as cell lines, patient-derived epithelial cultures (primary cultures), and mouse models are in use. All of these models have their own advantages and limitations. These models are discussed in brief in the next section.

1.6 Models for investigating prostate biology:

1.6.1 The primary patient-derived prostate epithelial culture model:

In our lab, most of the investigations rely heavily on patient-derived primary prostate epithelial cultures (PPECs) with supplementary use of cell lines and mouse xenografts established from patient-derived tissue. PPECs provide a near-to-patient model for investigating prostate epithelial cells from benign or malignant origin. However, there are several limitations to PPECs: (i) it is difficult to establish cultures from patient samples, (ii) these cultures have a limited life span (about 10 passages

in culture), (iii) cells show increasing cultural adaptation with each increasing passage such increase cell surface area, (iv) they are relatively difficult to manipulate (e.g. transfection) and (v) the luminal cells cannot be cultured. In spite of these limitations, PPECs can more faithfully replicate patient prostate scenarios (Peehl, 2005) and the luminal cells can still be investigated by their isolation prior to culture. We utilised CD133, CD44, CD24, and $\alpha_2\beta_1$ integrin as cell surface markers to enrich prostate epithelial subpopulations (Collins et al., 2001, Richardson et al., 2004, Collins et al., 2005) (**Figure 1.7**). The same marker profile was used for the isolation of cell sub-populations from PPECs derived from cancer and benign patient samples.

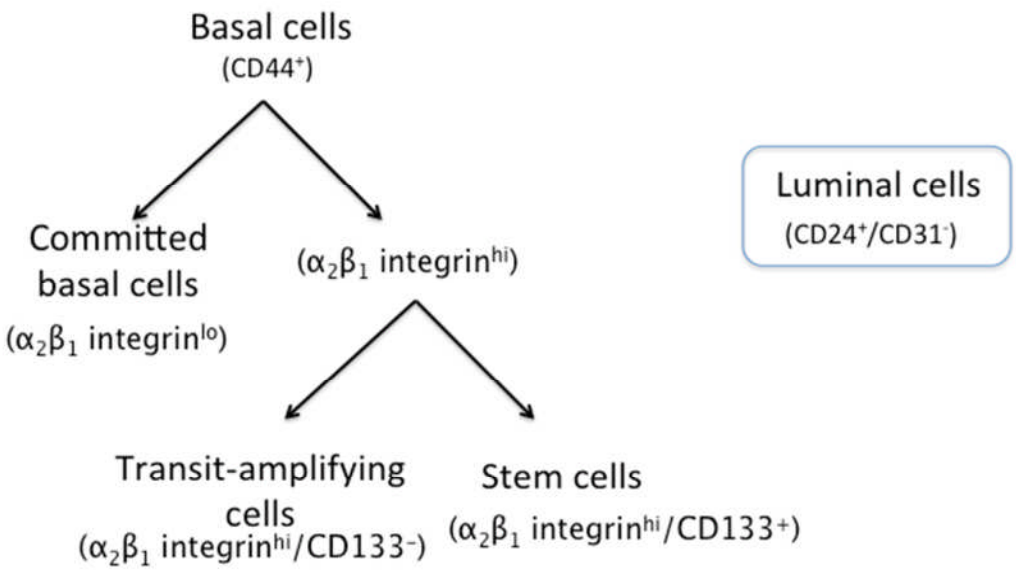


Figure 1.7: Enrichment of prostate epithelial sub-populations from primary prostate epithelial culture.

Characteristics of PPECs:

Features of stem cells (benign and malignant) enriched from PPECs:

- Rare (< 0.01%) (Richardson et al., 2004, Collins et al., 2005)
- Pluripotent (Richardson et al., 2004, Collins et al., 2005)

- Self-renewal (Richardson et al., 2004, Collins et al., 2005)
- Quiescence (Frame et al., in preparation)
- Radioresistant and chemoresistant (Frame et al., in preparation, Klein et al., in preparation)
- Distinct mRNA levels profile (Birnie et al., 2008)
- Higher heterochromatin content compared to differentiated cells (Frame et al., in preparation)

Features of cancer-derived cultures/tissue (compared to BPH-derived cultures):

- More proliferative and invasive (Collins et al., 2005)
- Distinct mRNA levels (Birnie et al., 2008)
- Exhibit characteristic TMPRSS2-ERG fusion in 50% of the cases (Polson et al., submitted)
- Form subcutaneous (Maitland et al., 2011) and orthotopic (Collins et al., in preparation) xenografts in immunocompromised mice

The presence of these characteristic features confirms that the majority of cells in cancer biopsies are indeed prostate cancer cells and we can reliably enrich prostate epithelial stem cells.

1.6.2 The cell line models of prostate:

Almost all the techniques used in this project were first validated in a panel of prostate cell lines. The cell lines also provide an attractive model for investigating molecular and cellular mechanisms in detail. The cell lines used in this project are summarised in **Table 1.5**.

Cell Line	Origin	Method of immortalisation	Reference
PNT1a	Normal prostate cells	SV40 large T antigen	(Berthon et al., 1995)
PNT2c2			
BPH-1	BPH tissue	SV40 large T antigen	(Hayward et al., 1995)
P4E6	Gleason 4, organ confined prostate cancer	human papillomavirus-16 *E6* gene	(Maitland et al., 2001).
RC165N/ hTERT	Primary benign tissue for a patient with prostate cancer	Overexpression of telomerase via hTERT transfection	(Miki et al., 2007).
R92a-N/hTERT	Primary prostate cancer, non-metastatic	Overexpression of telomerase via hTERT transfection	(Miki et al., 2007).
PC346C	Primary prostate cancer, non-metastatic	Selection after growing as a xenograft, retroviral immortalisation	(Marques et al., 2006)
LNCaP	Prostate cancer lymph node metastasis	Spontaneous immortalisation	(Horoszewicz et al., 1980)
VCaP	Prostate cancer brain metastasis	Cells were passaged in SCID mice and then established as a continuous in vitro cell line	(Korenchuk et al., 2001)
DU145	Prostate cancer brain metastasis (Patient also had leukemic brain metastasis)	Cells were passaged 60 times in nude mice and then established as a continuous in vitro cell line	(Mickey et al., 1977)
PC3	Prostate cancer bone metastasis	Spontaneous immortalisation	(Kaighn et al., 1979)

Table 1.5: Commonly used prostate epithelial cell lines.

1.6.3 Animal models for prostate cancer:

There are two types of animal models: (1) xenograft mouse models: human prostate cancer tissue is transplanted subcutaneously or orthotopically in immunocompromised mouse (Liu et al., 1996, Maitland et al., 2011) and (2) transgenic rodent prostate cancer models: prostate specific mutation in one or more key genes (e.g. PTEN) involved in prostate carcinogenesis leading to rodent

prostate cancer (Shen and Abate-Shen, 2010). These in vivo models provide a perspective of epithelial growth in relation with surrounding stroma. They also provide a good model for lineage tracking of cells to determine cell of origin of cancer and the stem cell phenotype (Lawson et al., 2010, Choi et al., 2012, Ousset et al., 2012). However, differences in rodent and human prostates must also be considered before interpreting results obtained from transgenic mouse models: (i) rodents do not develop spontaneous prostate cancer (Shen and Abate-Shen, 2010), (ii) distinct lobes present in the rodent prostate are absent in the human prostate (Roy-Burman et al., 2004), (iii) the rodent prostate does not exhibit classical bilayered epithelial architecture (El-Alfy et al., 2000, Roy-Burman et al., 2004), and (iv) stroma is sparse in the rodent prostate (Tsujimura et al., 2002).

This thesis is divided into three sections:

- Section I: The genetic regulation of prostate epithelial differentiation
- Section II: The identification of the role of telomerase in BPH and prostate cancer
- Section III: miRNA profiling of prostate epithelial sub-populations

The specific introduction for each section is described at the beginning of respective section.

2. AIMS AND OBJECTIVES

Two main objectives are driving prostate cancer research at this moment: (i) biomarker discovery - identification of biomarkers, which can distinguish between indolent and aggressive prostate cancer, early diagnostic biomarkers, and biomarkers that can be used to evaluate therapy response; and (ii) the design of novel therapeutic strategies, which could be useful for the management of advanced prostate cancer. In this project, we focussed on the second problem with specific emphasis on the cancer stem cell hypothesis, which proposes that the relatively rare cancer stem cells are the principal drivers of the cancer progression and should be specifically targeted for more efficient cancer management. Our lab has established a technique to enrich for prostate cancer stem cells and their differentiated progeny (transit amplifying cells, committed basal cells, and luminal cells) from patient-derived tissue material. Utilisation of these sub-populations enabled us to design experiments on tissue material closest to that of patients, focussing on individual prostate epithelial cell sub-types. With these pure and homogeneous prostate epithelial sub-populations, we wanted to investigate specific genetic and epigenetic (miRNA mediated) regulatory machineries essential for prostate cancer stem cell maintenance. Identification of such pathways would permit designing of prostate cancer stem cell-targeted therapeutic/diagnostic/prognostic approaches.

2.1 Objectives:

1. Identification of transcription factors, which can regulate prostate cancer stem cell differentiation.
2. Investigation of the role of telomerase in the maintenance of prostate cancer stem cells

3. Establishment of a miRNA profile for prostate epithelial sub-populations enriched from PrEC, BPH, treatment naïve cancers, and castration resistant prostate cancers, which can subsequently be exploited for the identification of therapeutic/diagnostic/prognostic miRNA candidate targets.

3. MATERIALS AND METHODS

3.1 Bioinformatic analyses:

3.1.1 mRNA microarray data reanalysis

The previously published microarray data from our lab (Birnie et al., 2008) was reanalysed by robust multi-array averaging (RMA) as opposed to MAS5.0 in the original paper. The gene expression was plotted as log2 expression. The expression in different prostate epithelial sub-populations was compared using a paired two-tailed t-test. Dr. Alastair Droop performed this analysis.

3.1.2 Promoter analysis:

The MatInspector software suite from Genomatix was utilised to identify potential binding sites for transcription factors in the promoters of candidate genes (Quandt et al., 1995, Cartharius et al., 2005). MatInspector is a tool, which utilises a library of matrix descriptions for transcription factor binding sites to locate matches in the promoter sequence. A large library of predefined matrix descriptions for transcription factor binding sites, based upon direct wet-lab confirmation data, exists within the software resource base. The software used a pre-defined sequence of a gene and promoter region, which is known or predicted to occupy binding sites for regulatory transcription factors.

3.1.3 Identification of CpG islands:

Regions of genomic sequence spanning 10,000 bp promoter and an entire genomic region of the LCN2, CEACAM6, S100p, and SPRR3 were analysed for the presence of CpG islands (regions that were rich in CG dinucleotides) The EMBOSS CpGPlot software was utilised for this analysis (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). The presence and location

of CpG islands fitting specific criteria within each gene and their promoters was determined:

- The observed-to-expected CpG ratio was greater than 0.6.
- The percentage of CG dinucleotides was greater than 50%.
- The CpG island was greater than 50 bp in length.

3.2 Models used for prostate epithelial analysis

Patient-derived primary prostate samples obtained from York District Hospital, York or Castle Hill Hospital, Hull were utilised for the majority of investigations. For each sample, the BPH or cancer diagnosis was confirmed first by histological examination. The patient sample collection (TURP or radical prostatectomy) was carried out as per the guidelines mentioned in an ethical permission (R0609-07/H1304/121) granted to Prof. Norman Maitland with informed consent from patients.

3.2.1 Establishment of patient-derived prostate epithelial cultures (primary cultures)

3.2.1.1 Processing of patient-derived tissue sample

Epithelial cultures from primary prostate samples were established according to the protocol established in our lab (Richardson et al., 2004). Before processing, a piece from each sample was snap frozen [in OCT (Sakura Finetech)] and then stored at -80^{0}C. Another piece was fixed in 10% formalin and paraffin embedded (for histological and molecular analysis, if necessary). Part of some of the cancer samples was kept aside for xenografting. The remaining tissue was then chopped into fine pieces (each piece less than ~ 3x3 mm) with a scalpel and subjected to overnight collagenase I (Lorne Laboratories) treatment at 37^{0}C in 2.5 ml

Keratinocyte Serum-Free Medium (KSFM with 5 ng/ml human EGF and 50 µg/ml bovine pituitary extract supplements (Invitrogen) and 5 ml Roswell Park Memorial Institute-1640 medium (RPMI, Invitrogen), supplemented with 10% foetal calf serum (PAA Laboratories Ltd.), 100 U/ml antibiotic/antimycotic solution and 2 mM L-Glutamine (Invitrogen) with shaking at 80 RPM. When luminal cell selection was planned, the above mixture was additionally supplemented with 10nM dihydrotestosterone (DHT) (Sigma).

The next day, the digestion was triturated by passing through a 5ml syringe (BD Biosciences) with a 21G blunt needle (Monoject) and cells collected by centrifuging at 2000 RPM for 10 minutes. The supernatant was removed and the pellet was washed twice with 10 ml of PBS to remove collagenase. Then, the pellet was suspended in 10 ml of R10 (RPMI media supplemented with 10% foetal calf serum (FCS) (PAA) and 2mM L-Glutamine), and both the stroma and the epithelium was separated by multiple centrifugations at 800 RPM for 1 minute. Stromal cells float in the supernatant while the epithelium (acini) form a loose pellet at the bottom. Stromal cell separation was not done with cancer core biopsies as we noted that there was extremely limited amount of stroma in them and in an attempt to separate it, we used to significantly lose epithelial acini. The pellet containing epithelial acini was then washed with 10 ml of PBS and centrifuged at 1800 RPM for 5 minutes. The supernatant was discarded and the pellet was further disrupted by suspending it into 5ml of 0.05% (v/v) trypsin-EDTA in PBS (Invitrogen) for 30 minutes at 37^0C at 80 RPM. Trypsin was then neutralised by 10 ml of R10 and the pellet containing prostate epithelial cells was collected by centrifuging the mix at 1400 RPM for 4 minutes.

The pellet was then suspended in 5 ml of stem cell medium (SCM) composed of Keratinocyte Serum-Free Medium, 5 ng/ml human EGF, 50 µg/ml bovine pituitary extract, 2 ng/ml leukaemia inhibitory factor (Chemicon), 100 ng/ml cholera toxin

(Sigma), 1 ng/ml granulocyte macrophage colony stimulating factor (Miltenyi Biotec), 2 ng/ml stem cell factor (First Link UK Ltd) and 2 mM L-Glutamine. The cells in SCM were plated on 10 cm type 1 collagen-coated dishes (BD Biosciences) with irradiated mouse embryonic fibroblasts cells (STO) as a feeder layer. The cells in these cultures were of a basal prostate epithelial phenotype. These cells were incubated at 37^{o}C with 5% CO_2 and needed 2-3 weeks to reach the first confluency. Thereafter cells were sub-cultured by splitting at a ratio of 1:3 on a weekly basis. However, there is a significant heterogeneity in the growth kinetics of samples. These prostate epithelial cells could be maintained in culture for about 10 passages. Similar cultures are referred to as **primary cultures or primary prostate epithelial cultures (PPECs)** in further description. The normal prostate epithelial cells, PrEC cells (Lonza), were cultured in SCM and maintained in a similar fashion as prostate epithelial cells established in our lab.

3.2.1.2 Enrichment of hierarchical sub-populations from primary cultures:

Four sub-populations at different differentiation stages can be enriched from prostate epithelium. The luminal cell sub-population, which can't be cultured, was enriched before cells were plated in type1 collagen-coated plates. A CD24 indirect magnetic-activated cell sorting (MACS) method (Miltenyi Biotec) was utilised to enrich $CD24^{+}$ luminal cells, using the manufacturer's protocol during this enrichment. The other three populations [committed basal cells (CB), transit-amplifying cells (TA), and stem cells (SC)] were enriched from primary cultures, although they can be enriched from uncultured epithelium as well (if they are enriched from uncultured epithelium, the number of stem cells is usually <1000 cells: not sufficient for most of the experiments). The majority of cells used in this investigation were cultured for 2 passages after which SC, TA, and CB cells were enriched.

Enrichment of cells expressing higher levels of $\alpha_2\beta_1$ integrin was performed by a rapid collagen adhesion method. Cells were grown up to 80% confluency and harvested with 0.05% trypsin-EDTA. Type1 collagen-coated 10 cm plates were blocked with 0.3% BSA (0.3% bovine serum albumin in PBS, heat-denatured at 80°C for 5 min) for 1 hour at 37°C. Harvested cells from up to three 80% confluent plates were suspended in 3 ml of SCM and plated out on to blocked plates and incubated at 37°C for 20 minutes. Media was collected with the unattached cells and other loosely attached cells were removed by multiple PBS washes. Cells in the pellet collected from media and PBS washes were classified as $\alpha_2\beta_1^{lo}$ committed basal cells. Cells attached to blocked collagen coated plates ($\alpha_2\beta_1^{hi}$) then trypsynised and subjected to further CD133 selection.

The direct CD133 Cell Isolation Kit (Miltenyi Biotec) was used to enrich $CD133^+$ cells from $\alpha_2\beta_1^{hi}$ cells. Up to 10^8 cells were re-suspended in 300 µl magnetic-activated cell sorting (MACS) buffer (2 mM EDTA, 0.5% (v/v) FCS in PBS), 100 µL FcR blocking reagent (Miltenyi Biotec) and 100 µL CD133 beads (Miltenyi Biotec) and incubated at 4°C for 30 min. Cells were then washed with 3 ml MACS buffer, centrifuged at 1500 RPM for 5 min and the pellet was re-suspended in 500 µl MACS buffer. Separation of CD133 expressing cells on MACS MS columns was then performed according to the manufacturer's instruction. The $CD133^+$ cells were collected first by passing CD133 labelled $\alpha_2\beta_1^{hi}$ cells through MS MACS columns. CD133 cells collected from columns were further enriched by passing them again through another MS MACS column. This dual column use ensures about 95% purity of $CD133^+$ populations. Cells that are not labelled for CD133 in this selection were classified as $CD133^-\alpha_2\beta_1^{hi}$ transit amplifying cells. Thus we could enrich four distinct prostate epithelial subpopulations (**Table 3.1**). These cells were plated out or used to collect RNA/proteins for further analysis.

Cell sub-population	Surface marker profile
Stem cells (SC)	$CD133^{+}\alpha_2\beta_1^{hi}$
Transit amplifying cells (TA)	$CD133^{-}\alpha_2\beta_1^{hi}$
Committed basal cells (CB)	$CD133^{-}\alpha_2\beta_1^{lo}$
Luminal cells (LC)	$CD24^{+}$

Table 3.1: Surface marker profile used to enrich prostate epithelial sub-populations from primary patient prostate samples (Richardson et al., 2004, Collins et al., 2005).

3.2.1.3 Enrichment of hierarchical sub-populations from xenografts:

A similar method was used to enrich prostate epithelial sub-populations from xenografts (Maitland et al., 2011). Dr. Anne Collins with assistance from Mr. Paul Berry from our lab has established a patient-derived xenograft model where, a prostate tissue or a specific sub-population of epithelial cells was directly placed at a sub-cutaneous location in immunocompromised $Rag2^{-/-}\gamma C^{-/-}$ mice. The $Rag2^{-/-}\gamma C^{-/-}$ mice are severely immunocompromised mice, which also lack natural killer cell activity. Before enriching prostate epithelial sub-populations using the surface markers as mentioned in **Table 3.2**, mouse cells and endothelial cells were depleted by removing cells expressing mouse haematopoietic lineage markers (Lin^{+} cells) and an endothelial marker ($CD31^{+}$ cells) using MACS sorting. These cells do not demonstrate rapid collagen adhesion properties (most probably due to adaptation necessary for survival in a xeno-environment). Therefore $\alpha_2\beta_1$-integrin selection cannot be employed for xenograft-derived cell sorting.

Cell sub-population	Surface marker profile
Stem cells (SC)	$CD133^{+}$
Transit amplifying and basal cells	$CD44^{+}$
Luminal cells (LC)	$CD24^{+}$

Table 3.2: Surface marker profile used to enrich prostate epithelial sub-populations from xenografts. The MACS sorting method as described for primary culture fractionation was also used for enrichment of xenograft sub-populations.

3.2.1.4 Preparation of STO feeder cells:

STO cells were used as a feeder layer in primary cultures. These cells are mouse embryonic fibroblasts and were inactivated by γ-irradiating them with a dose of 60 Gy. Cells could be stored at 4°C for up to 5 days before use. Caty Hyde, Sandra Klein, or Richard Bingham prepared STO feeder cells.

3.2.2 Maintenance of prostate cell lines

Established cell lines were purchased either from the American Type Culture Collection (ATCC, USA) or the European Collection of Animal cell culture (ECACC, UK), except for PNT1A, PNT2C2 and P4E6 cells, which were established in our laboratory. PC346C cells were kindly provided by Dr. Robert Kraaij (Erasmus Medical Centre, The Netherlands). BPH-1 cells were kindly given by Dr. Simon Hayward and RC165N/hTERT and RC92a/hTERT by Prof. John Rhim. All the plasticware used in cell culture was purchased from Corning and cells were cultured at 37°C with 5% CO_2. All the cell lines were regularly DNA fingerprinted and checked for MYCoplasma contamination to ensure their suitability for experiments (by Hannah Walker and Paul Berry).

Table 3.3 below summarises the culture conditions for prostate cell lines used.

Cell line	Origin	Culture media	Approx. freq. of sub-culture	Maximum passage
PNT2c2	A sub-clone of PNT2 cells (PNT2 cells were prepared by immortalisation of normal prostate cells by transfection with SV40)	**R10** (RPMI media supplemented with 10% foetal calf serum (FCS) and 2mM L-Glutamine)	3-4 days	130
PNT1a	Normal prostate epithelium immortalised with SV40	**R10**	3-4 days	80

BPH-1	Benign prostatic hyperplasia	**R5** (RPMI media supplemented with 5% foetal calf serum (FCS) and 2mM L-Glutamine)	3-4 days	70
P4E6	Early stage well-differentiated prostate cancer immortalised by retroviral delivery of the HPV16-E6 gene	**K2** (Keratinocyte Serum-Free Medium, 5 ng/ml human EGF, 50 µg/ml bovine pituitary extract and 2mM L-Glutamine with 2% FCS)	A week	55
RC165N/hTERT	hTERT-immortalised primary benign prostate tissue	**KSFM** (Keratinocyte Serum-Free Medium, 5 ng/ml human EGF, 50 µg/ml bovine pituitary extract and 2mM L-Glutamine)	A week	40
RC92a/hTERT	hTERT-immortalised primary human prostate tumour	**KSFM**	A week	40
PC-3	Human prostatic adenocarcinoma metastatic site in bone	**H7** (Ham's F-12 medium + 7% FCS + 2mM L-Glutamine)	4-5 days	50
LNCaP	Human prostatic adenocarcinoma metastatic site in supra-clavicular lymph node	**R10**	A week	50
VCaP	Mouse xenograft established from hormone refractory prostate cancer bone metastasis	**R10**	10 days	80
DU145	Human prostatic adenocarcinoma metastatic site in brain	**R10**	A week	150
PC346c	Human prostate carcinoma xenograft	1:1 mix of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium, 100 µg/ml streptoMYCin, 100 U/ml penicillin G, 2% FCS, 0.01% (w/v) BSA (Sigma), 10 ng/ml EGF (Sigma), 1% (v/v)	2-3 weeks	Upper limit not assigned

		ITS-G (GIBCO), 0.1 nM R1881 (DuPont-New England Nuclear), 1.4 µM hydrocortisone (Sigma), 1 nM triiodothyronine (Sigma), 0.1 mM phosphoethanolamine (Sigma), 50 ng/ml cholera toxin (Sigma), 0.1 µg/ml fibronectin (Sigmg/ml fetuin (Sigma)		

Table 3.3: Prostate epithelial cell lines used in this investigation.

3.2.3 Cryopreservation of mammalian cells

For long-term storage in liquid nitrogen, cells were collected after trypsinisation. A standard 1 ml freezing media (10% DMSO and 20% FCS in RPMI) was used to re-suspend cells (~1-2 x 10^6) in cryovials. These vials were placed in containers at -80^{o}C for 4 hours and then transferred into liquid nitrogen. While thawing cells for culture, vials were thawed as quickly as possible by placing it into a 37^{o}C water bath and then the growth media was added to dilute the freezing media drop by drop. This was to avoid rapid change in cellular osmolality, which can affect cell integrity. Cells were then pelleted by centrifugation at 1300 RPM for 4 minutes and plated in an appropriate media.

3.2.4 Live cell counting

In order to determine live cell count, 30µl of cell suspension was added to an equal volume of diluted Trypan Blue stain (Sigma-Aldrich). The live cells were counted using a haemocytometer (Neubauer). Total live cell count was determined by the following formula:

Total number of cells/ml of cell suspension = (Total no. of cells in 4 quadrants/4) * 10,000

3.2.5 Foetal calf serum hormone depletion

2 g of Norvid A charcoal (Sigma) was added to 100 ml FCS and incubated at 4°C overnight. The mixture was centrifuged at 5000 RPM for 10 min to sediment the charcoal, and the supernatant re-centrifuged repeatedly at 5000 RPM for 10 min until the supernatant cleared (about 5 centrifugations). The FCS was then filtered using a 0.2 µM filter and stored at 4°C until use. This process removes steroid hormones and other lipid-based hormones and growth factors.

3.3 Extraction and analysis of RNA

RNA analysis was routinely performed on freshly collected primary cells or cell lines and on cell pellets frozen at -80^{0}C.

3.3.1 RNA extraction

To ensure isolation of pure, uncontaminated RNA, bench and relevant equipments were cleaned with 70% ethanol. Dedicated filter tips were also used for RNA extraction purpose and samples were kept on ice all the time. RNA was extracted using either QIAGEN RNeasy Mini kit (QIAGEN) or the mirVana kit (Invitrogen).

3.3.1.1 The RNA extraction using QIAGEN RNeasy Mini kit

The QIAGEN RNeasy kit protocol utilises selective binding properties of a silica-based membrane and microspin technology with high-salt based system to purify RNA. Cells were lysed with specified amount of buffer by vortexing and then up to 350µl cell lysate was processed per column. First, the cell lysate was homogenised by passing it through QIAshredder column and then RNA was purified by serial buffer washes in QIAGEN RNeasy Mini columns. For maximum RNA elution, 30µl of nuclease free water (Sigma) was placed in a column and a column was centrifuged after 1 min. The same eluate was passed through the column again to collect

leftover RNA. On occasions, if the stem cell number was less than 2000, RNA was eluted in 15µl water.

3.3.1.2 The RNA extraction using mirVana kit

The mirVana kit utilises acid:phenol-chloroform extraction followed by immobilisation of RNA on glass-fibre filter to purify RNA. 300µl of cell lysate was used per column and total RNA was eluted in 30µL of 95^{o}C heated nuclease free water (Sigma) as per the kit protocol.

3.3.1.3 The preparation of concentrated RNA

Sometimes, if concentrated total RNA is required (eg. For miRNA microarray), total RNA was concentrated using ethanol precipitation method. The RNA sample was mixed with 0.1 volumes 3M-sodium acetate (pH 5.2) and vortexed for 10s. 2.2 volumes of cold 100% ethanol was added to it. After thorough mixing, samples were incubated at -80^{o}C for 2 hours. Later, the samples were centrifuged at 13,000 g for 10 minutes and supernatant was removed. The pellets were then re-suspended in cold 70% ethanol and spun at 13,000 g for 10 minutes. The ethanol was then removed and pellets were vacuum dried at 45^{o}C for 1 hour with a vacuum centrifuge concentrator (Eppendorf). The dried pellets were re-suspended in the desired quantity of RNase-free water.

3.3.1.4 The quality control of extracted RNA

The extracted RNA was quantified and its quality assessed, in all the cases, by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Only the RNA samples with optical density (OD) ratio of about 2.00 at A260/A280 and A260/A230 were used for future experiments. The A230/A260 ratio was sometimes very low for stem cells derived RNA, but these samples were still included in analysis if the ratio was above 1. This is because: (i) stem cells-derived RNA concentrations varied between 2-5ng/µl, which was at about the threshold of spectrophotometer detection

levels and at that level, the accuracy in determination of OD may vary significantly and (ii) sometimes, it was extremely difficult to get pure RNA from stem cells even after best possible precautions. After quality confirmation, the RNA samples were labelled and stored at -80^{o}C.

3.3.2 Preparation of cDNA from mRNA

3.3.2.1 Method 1:

First strand cDNA synthesis was carried out by mixing up to 2 µg of RNA, 100ng random hexamer primer (Invitrogen), 1µl of 10mM dNTPs (Invitrogen) in RNase free water (Sigma) per sample, making a total volume of 12µl. The mixture was incubated at 65^{o}C for 5 minutes, and snap cooled on ice for 2 minutes. Components shown in **Table 3.4** were added in the mix and the mix was incubated at 25^{o}C for 10 minutes, followed by 42^{o}C for 50 minutes, and the reaction was terminated at heating it 70^{o}C for 15 minutes in a PCR machine (GeneAmp PCR system 9700).

Component	Amount per sample
5x first strand buffer (Invitrogen)	4µl
0.1M DTT (Invitrogen)	2µl
RNaseOUT (Invitrogen)	1µl
Superscript II (Invitrogen)	1µl
Total	8µl

Table 3.4: Components used to prepare cDNA

The cDNA fragments obtained were then purified using QIAquick PCR Purification Kit (QIAGEN). This kit applies a silica-based membrane, which removes nucleotides, mineral oil, and other impurities from DNA. The DNA was eluted in 30µL of nuclease free water. The prepared cDNA was quantified and analysed by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) similar to that of RNA. The cDNA samples were labelled and stored at -20^{o}C.

3.3.2.2 Method 2:

At times, when the starting RNA amount was lower (e.g. for stem cells); a different method was employed to prepare cDNA, which involved an ethanol precipitation step. In this method, about 50ng of RNA was diluted in 15µl of nuclease free water and 0.75µl of random hexamer primers (3µg/µl, Invitrogen) were added per sample. The mix was incubated at 70^{o}C for 10 min. and then snap cooled on ice for 5 min. A reaction mix as shown in **Table 3.5** was prepared and added to each sample reaction, which was then incubated at 45^{o}C for 2 hrs. Thereafter, using serial ethanol precipitation, first by pre-cooled analytical grade 100% ethanol and then by 70% pre-cooled ethanol, cDNA was resuspended in 30 µl of nuclease free water.

Component	Amount per sample
Water	0.75µl
5x first strand buffer (Invitrogen)	6µl
0.1M DTT (Invitrogen)	3µl
10mM dNTPs (Invitrogen)	3µl
Superscript III (Invitrogen)	1.5µl
Total	14.25µl

Table 3.5: Reaction mix for ethanol precipitation method for cDNA preparation.

It was noted that the ethanol precipitation method needed significantly more reagents, but it also gave reliable, reproducible, and higher cDNA yields.

3.3.3 Quantitative reverse-transcriptase PCR (qRT-PCR)

In a 96-well plate, a 10µl PCR reaction was prepared using constituents as shown in **Table 3.6**. Each sample was assayed in triplicate with non-template control and water only control for each gene. The plate was sealed with thermoclear film lid and a qRT-PCR was performed using ABI-70000/7700 (Thermal profile: **Table 3.7A**) or Bio-Rad C1000 thermal cycler (Thermal profile: **Table 3.7B**). The probes used in this process were described in **Table 3.8**.

Component	Amount per sample
2X Master mix (ABI/Biorad)	5µl
20X TaqMan assay mix (Applied Biosystems)	0.5µl
cDNA + water	4.5µl
Total	10µl

Table 3.6: Reaction mix for qRT-PCR

A	Temperature (^{o}C)	Duration
1	50	15 min
2	95	2 min
3	95	15sec
4	60	30 sec
40 cycles for step (3+4)		

B	Temperature (^{o}C)	Duration
1	95	2 min
2	95	5 sec
3	60	5sec
40 cycles for step (2+3)		

Table 3.7: Thermal profile for ABI thermocycler (**A**) and for Bio-Rad thermocycler (**B**) for qRT-PCR.

Gene	TaqMan Probe ID
LCN2	Hs00194353_m1
CEACAM6	Hs00366002_m1
S100p	Hs00195584_m1
SPRR3	Hs00271304_m1
RPLP0	Hs99999902_m1
hTERT	Hs00972656_m1

Table 3.8: TaqMan gene probes used for qRT-PCR (Applied Biosystems).

The gene expression was quantified relative to RPLP0 internal control gene using either the ddCt method (Livak and Schmittgen, 2001) or the standard curve method (Larionov et al., 2005).

3.3.4 Quantitative reverse-transcriptase PCR (qRT-PCR) for miRNA

For miRNA qRT-PCR, total RNA from cells was extracted using mirVana kit. TaqMan small RNA assay was employed for qRT-PCR that uses a stem-looped primer for reverse transcription and amplification of an individual miRNA and a sequence specific TaqMan probe to detect mature miRNA. Reverse transcription

(RT) was performed using TaqMan MicroRNA Reverse Transcription Kit employing manufacturer's protocol (Life Technologies). The RT mix was prepared as described in **Table 3.9** and incubated on ice for 5 minutes and placed in a thermal cycler programmed as described in **Table 3.10**.

Component	Master mix volume per 15-µL reaction
100mM dNTPs (with dTTP)	0.15 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µL
10× Reverse Transcription Buffer	1.50 µL
RNase Inhibitor, 20 U/µL	0.19 µL
Nuclease-free water	4.16 µL
5X RT primer	3.00 µL
Total RNA (1-10ng)	5.00 µL
Total volume	**15.00 µL**

Table 3.9: Component of RT master mix for miRNA reverse transcription reaction

Step	Time	Temperature (^{o}C)
Hold	30 min	16
Hold	30 min	42
Hold	5 min	85
Hold	∞	4

Table 3.10: Thermal profile for miRNA reverse transcription reaction

The reverse transcription reaction final product was used to determine expression of miRNA under consideration. The reaction mix for qPCR was prepared as mentioned in **Table 3.11** and the plate was loaded in a thermocycler using the protocol described in **Table 3.12**.

Component	Volume (µL)
TaqMan® Small RNA Assay (20×)	1
Product from RT reaction	1.33
TaqMan® Universal PCR Master Mix II (2×), no UNG	10.00
Nuclease-free water	7.67
Total	20

Table 3.11: Components of miRNA qPCR reaction mix.

Step	Temperature (^{o}C)	Time
Enzyme activation	95	10 min
PCR (40 cycles)	95	15 s
	60	60 s

Table 3.12: Thermal profile for miRNA qPCR reaction.

The dCt method was used to analyse qPCR data and the expression was plotted relative to the expression of an internal control (RNU6b).

3.4 Protein analysis

Results obtained at the RNA level were confirmed at protein levels by various protein expression assays.

3.4.1 Extraction of proteins

Freshly harvested cells or cell pellets stored at -80^{o}C were used to extract proteins. An appropriate amount of CytoBuster (Novagen) was added and the mixture was incubated at room temperature for 5 minutes. If pellets were used to extract proteins, after adding CytoBuster, the pellet was dissociated by brief vortexing. The extract was centrifuged for 5 min at 16,000 x g at 4^{o}C and aliquoted in 50 µl tubes with 1X protease inhibitor (Roche).

3.4.2 Measurement of protein concentration

Protein concentration was measured using Bicinchoninic acid assay (BCA) protein assay kit (Thermo Scientific) following the manufacturer's instructions. 25 µl of BSA standards and samples (unknown) were pipetted in triplicate into a 96 well plate. After adding 200 µl of working reagent to each well, the plate was mixed on a plate shaker for 30 seconds and then incubated at 37°C for 30 min. After cooling the plate to room temperature, the absorbance was measured at 562 nm on a POLARstar OPTIMA microplate reader (BMG Labtech). The protein concentration in the

unknown samples was determined according to the BSA standard curve of concentration vs. absorbance.

3.4.3 Western blot analysis

Western blot analysis was performed using 10 or 12% polyacrylamide gels. Protein lysate (usually 20µg) was mixed with 4X SDS loading buffer (10% (v/v) glycerol, 62.5 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 65 mM DTT and bromophenol blue to colour) in 1:4 (v/v) ratio. The mixture was briefly vortexed for 10 seconds and heated to 100°C for 15 min in a QBD2 heating block (Grant). After re-vortexing and centrifugation for 10 seconds each, samples were cooled on ice for 2 minutes and loaded (maximum volume of 50 µl) onto a 10-12% Tris-SDS polyacrylamide gel, using the Bio-Rad Protean II system. For each gel, a single marker lane was also run with 5 µl of Kaleidoscope Pre-stained Standards (Bio-Rad) and 2 µl of Bioatenylated Protein Ladder (Cell Signaling). The samples were then run on a gel, first at 60V for 30 min and then 80-90V for about 2 hours in SDS running buffer (25 mM tris, 0.19 M glycine and 3.5 mM SDS). In order to transfer proteins from the gel to an Immobilon-P membrane (Millipore) membrane (pre-activated in methanol and equilibrated in a transfer buffer), a transfer cassette was assembled with a gel and a membrane sandwiched between pre-soaked Whatmann filter papers and ScotchBrite foam pad. This assembly was run overnight at 4^{o}C at 40V or for 1hr at RT at 100V in a transfer buffer (48 mM tris, 39 mM glycine and 10% (v/v) methanol). While assembling a transfer cassette, special attention was given to remove any air-bubble trapped between various layers. If necessary, the membrane was dried and stored at 4^{o}C between filter papers for later use.

Membrane with freshly transferred proteins or previously air-dried membranes were re-wet with methanol and washed twice in TBS (150 mM NaCl and 50 mM Tris-HCl at pH 7.4) and then probed for required proteins. All the washes and incubations during this process were performed on a rocker. The membranes were first blocked

with 1% Marvel in TBST buffer (150 mM NaCl, 50 mM Tris-HCl and 0.1% (v/v) Tween-20, pH 7.4) for 1hr at RT. The primary antibody was added in 1% Marvel and the membranes were incubated in a plastic sandwich box (**Table 3.13**). After washing 4 times in TBST, membranes were incubated with the respective peroxidase-labelled secondary antibodies (1:5000 (v/v); Cell Signalling Technologies/Boehringer) and 1:5000 (v/v) anti-biotin-HRP (Cell signalling technologies) diluted in 1% (w/v) marvel in TBST were added for 1 hour at RT. After another washing step (4x, 10 min each), the strength of the signal was detected using chemiluminescence method with Kodak®BioMax^{TM} system (GBX developer, GBX fixer) and Amersham Hyperfilm ECL (GE Healthcare).

Primary antibody	Species	Origin	Working dilution	Incubation time
LCN2	Mouse	Abcam - ab23477	1:50	overnight
CEACAM6	Rabbit	Abcam - ab56234	1:1000	overnight
S100p	Mouse	BD-610307	1:100	overnight
SPRR3	Mouse	Abcam - ab58233	1:100	overnight
SPRR3	Rabbit	Sigma - HPA044467-100UL	1:100	overnight
β-actin	Mouse	Sigma	1:5000	1 h

Table 3.13: Antibodies utilised in western blot analysis.

3.4.4 Immunofluorescence

In order to obtain further information about the quantification of Ki67 protein expression, immunofluorescence was performed using 8-well collagen 1 coated slides (BD Biosciences). About 5000 cells were plated per well in 200 µl media. The next day, cells were washed with PBS twice and fixed in 4% PFA in PBS for 15 min. After 3 PBS washes, cells were permeabilised using 1% Triton-X in PBS for 10 minutes and blocked with 200ul of blocking solution [10% goat serum in PBS] for 1

hour. Later, the cells were incubated at 4^{o} C overnight with primary antibody in 10% goat serum solution in PBS (**Table 3.14**). The next morning, after 0.5% BSA/PBS washes, secondary antibody in 10% goat serum in PBS was added for 1 hour at RT. Slides were mounted using Vectashield with DAPI (Vector laboratories), which also counterstained cell nuclei. Slides were stored at 4°C until visualisation on a Nikon Eclipse TE300 fluorescent microscope (Nikon).

Experiment	1^{o} antibody	2^{o} antibody
Ki-67 staining	ab16667 (1:500), Abcam (1 hour RT)	Alexa Fluor 568 goat anti—rabbit (1:1000), Invitrogen

Table 3.14: Concentrations of Ki-67 antibody used for immunofluorescence.

3.5 Senescence staining for acidic β-galactosidase:

To identify senescent cells after telomerase inhibition, β-galactosidase staining of cells was performed. The senescence β-galactosidase staining kit (Cell Signaling) detects β-galactosidase activity at pH 6, which is a characteristic of senescent cells. Along with β-galactosidase staining, changes in cell morphology were also noted to corroborate the presence of senescence.

To stain for β-galactosidase, cells were washed with PBS and fixed with 1x fixative solution (10X solution: 20% fomaldehyde, 2% glutaraldehyde in 10X PBS, further diluted with distilled water to prepare 1X solution) from the kit for 10-15 min at RT. Fixative solution was washed with PBS and the cells were incubated at 37^{o}C overnight in a dry incubator with β-galactosidase staining solution at pH 6: for each 35 mm well to be stained, the following was combined in a polypropylene container to prepare a staining solution:

a. 930 µl 1X Staining Solution (10x solution: 400 mM citric acid/sodium phosphate (pH 6.0), 1.5 M NaCl, 20 mM $MgCl_2$, diluted with distilled water to prepare 1X solution)

b. 10 µl Staining Supplement A (500 mM potassium ferrocyanide)

c. 10 µl Staining Supplement B (500 mM potassium ferricyanide)

d. 20 µl 50 mg/ml X-gal (Promega)

Cells were then observed at either 10X, 20X, or 40X magnifications for the development of blue colour with an Evos light microscope (AMG).

3.6 Colony forming assay:

A colony forming assay or clonogenic recovery assay was performed to determine self-renewing capacity of cells. This assay is a primary indicator of the stem cell characteristics of a given cell type. After transfecting cells with test (hTERT) and control siRNA for 7 days, the cells were trypsinised and about 200 cells were plated in each well of the 6-well Collagen-I coated plates (BD Biosciences). These cells could either be a whole population, or the selected epithelial sub-populations and were plated in triplicates with 2 ml of stem cell media and 500 µl of irradiated STO feeder cells per well. The cells were then incubated at 37^{o}C and the medium was changed every 2-3 days. Clonal colonies were counted at the end of a suitable end point (usually 10-15 days, when the observed colonies were of a significant size of about >32 cells, representing more than 5 population doubling).

3.7 Transfection of cells with siRNAs

Transient (up to a week) loss-of-function studies were performed using siRNA mediated gene knock-down. siRNAs predominantly inhibit protein translation and promote mRNA degradation. siRNAs (Applied Biosystems) were aliquoted in 50nM final concentration and stored at -80^{o}C. For control, Applied Biosystems control siRNA #5 was used (In a test analysis conducted by Dr. Richard Birnie at ProCure therapeutics Ltd. with PPECs and control siRNAs, control siRNA #5 was found to be

the one that least influenced cell viability and gene expression. On day 1, cells were plated in a relevant growth media (generally, ~20000 cells/cm^2) and incubated overnight at 37^oC (**Table 3.15**).

	96-well plate	24-well plate	6-well plate	10 cm dish
Cells/well	1 X 10^4	5 X 10^4	2 X 10^5	1.5 X 10^6
Media	0.1 ml	0.5 ml	2 ml	5 ml

Table 3.15: The number of cells plated and media used for siRNA experiments.

On day 2, in separate tubes for 50nM final siRNA concentration, appropriate amount of siRNA (Tube 1) and the appropriate amount of Oligofectamine transfection reagent (Invitrogen) (Tube 2) were diluted in serum-free Opti-MEM serum free medium (Invitrogen) (**Table 3.16**). The contents in both tubes were mixed by pipetting and incubated for 10 min at RT. Then the contents from both tubes were mixed with each other and incubated for 25 min at RT. Cells were washed with Opti-MEM media and incubated at 37^oC in 5% CO_2 with the siRNA/Oligofectamine mix for 4 hours. Growth media was added and cells were incubated under the same conditions for 4 more hours. Later, the transfection mix was aspirated and cells were washed twice with 1X PBS and then incubated in the growth media for required time.

	Tube 1	**Tube 2**	**Wash**	**Opti-MEM**	**siRNA/ Oli**	**Media**
96 well	0.2 µl siRNA + 25 µl Opti-MEM	0.2 µl Oli + 6.8 µl Opti-MEM	100 µl	17.5 µl	32.5 µl	200 µl
24 well	0.5 µl siRNA + 62.5 µl Opti-MEM	0.5 µl Oli + 17 µl Opti-MEM	250 µl	44 µl	81 µl	500 µl
6 well	2 µl siRNA + 250 µl Opti-MEM	2 µl Oli + 68 µl Opti-MEM	1 ml	175 µl	325 µl	2 ml
10cm	2 µl siRNA +250 µl Opti-MEM	16 µl Oli + 544 µl Opti-MEM	8 ml	1400 µl	2600 µl	5 ml

Table 3.16: siRNA reagents for 50nM siRNA transfection. SiRNA stock solution: 50nM.

3.8 miRNA transfection of cells

mirVana miRNA mimics were ordered from Applied Biosystems and used to transfect cells with the same protocol as for siRNA transfection.

3.9 Chemical treatment of prostate cell lines or primary prostate epithelium

Short-term (6hr-1week) treatments with chemical modifiers were performed in order to investigate specific effect of perturbation of certain pathway or regulatory mechanism on prostate epithelium (**Table 3.17**).

Chemical agent	Mechanism of action	Conc. / range (Routine conc.)	Duration	Special comment
5-Aza-2-deoxy-cytidine (Azt) treatment of prostate cell lines	Inhibition of DNA methylation	1μM	4 days	Concentration previously validated in the lab [by D. Pellacani (Pellacani et al., 2011) and E. Oldridge- PhD thesis 2012]
Trichostatin-A (TSA) treatment of prostate cell lines	Inhibition of histone acetylation	0.6μM	2 days	Concentration previously validated in the lab [by D. Pellacani (Pellacani et al., 2011) and E. Oldridge- PhD thesis 2012]
All-trans retinoic acid (Sigma) treatment of prostate cell lines and PPECs	Stimulation of RAR and RXR receptors	10-1000nM (100nM)	6 hrs-1 week	Cells lines grown in charcoal-stripped media
9-cis-retinoic acid (Sigma) treatment of PPECs	Selective stimulation of RXR receptors	100nM	6hrs-1 week	Cell lines grown in charcoal-stripped media
1,25-dihydroxyvitamin D3 (Sigma) treatment of PPECs	Stimulation of VDR receptors	10nM	6hrs-1 week	Cell lines grown in charcoal-stripped media

Table 3.17: Chemical agents used to assess pathway functioning.

3.10 Telomere repeat amplification protocol (TRAP) assay

A mini-TRAP assay, which can be performed with as few as 50 cells, was employed to assess telomerase enzyme activity in prostate epithelial hierarchical sub-populations. Either freshly selected sub-populations or snap-frozen cell pellets were used for this assay. TRAPEZE® RT Telomerase Detection Kit (Millipore) was used to determine telomerase activity by qRT-PCR. First, a standard curve was created by using serial dilutions of TSR8 (quantitation control template). For each sample, 2500 cells were suspended in 10µL CHAPS lysis buffer and incubated on ice for 30 min. The suspension was centrifuged at 12,000g for 20 min at 4^{o}C and the supernatant was used for further analysis. TSR8 serial dilutions, P4E6 and cells provided with the kit were used as a positive control and heat inactivated (85^{o}C for 15 minutes) test cell extract was used as a negative control. Samples were kept on ice all of the time.

The following reagents were used to set up qRT-PCR reaction (**Table 3.18**):

Reagent	Volume (µL
5X TRAPeze RT reaction mix	5.0
Titanium® Taq DNA Polymerase (Clontech: 639208) (5unnit/µL)	0.4
Nuclease free water	17.6
Sample/control	2.0
Total	25

Table 3.18: Reagents used to set up qRT-PCR reaction for TRAP assay.

The reaction was run on ABI PRISM® 7700 Real Time PCR System using following set up (**Table 3.19**):

Temperature (^{o}C)	Duration	Cycle
30	30 min	1 cycle
90	2 min	1 cycle
45 cycles		
94	15 s	
59	60 s	
45*	60 s	

* Temperature at which reading was taken

Table 3.19: Thermocycler profile setting used for TRAP assay.

The relative test sample telomerase activity was quantified by comparing its Ct value with that of TSR8 template serial dilutions (As recommended by the manufacturer).

3.11 miRNA microarray analysis:

A microarray experiment was performed to determine global miRNA expression in prostate epithelial hierarchical sub-populations enriched from normal (PrEC cells, 1), BPH (5), treatment naïve cancers (5), and castration resistant cancers (3). For this purpose, an Agilent human miRNA microarray Kit (V3), 8x15K (Agilent, G4470C), which has 866 human miRNA probes printed was employed. RNA from frozen cell pellets (Stored at -70^{o}C) was extracted using MirVana kit, as described in the RNA extraction section. Using NanoDrop 2000, the quality of RNA samples was tested.

For miRNA microarray, 'miRNA Microarray System with miRNA Complete Labeling and Hyb Kit' (Agilent) protocol was employed as suggested by the manufacturer. In order to distinguish significant biological data from processing issues, the labelling and hybridisation spike-in solutions were prepared and mixed with test RNA samples subsequently. These 'spike-in solutions' produce control labelled luminescent spots with known intensity. The analysis of intensity of these points enables the assessment of labelling and hybridization efficiency.

To prepare test samples for miRNA, the following protocol was used:

3.11.1 Step 1: Preparation of the labelling reaction:

This step involves the ligation of one Cyanine 3-pCp molecule to the 3' end of a RNA molecule with greater than 90% efficiency. After removing 5´ phosphates from RNA by calf intestinal phosphatase (to avoid self-ligation), the T4 ligase catalyses this ligation.

2µl of total RNA (50ng/µl) was added to 2µl calf intestinal alkaline phosphatase (CIP) master mix (**Table 3.20**). The total mix of 4µl was mixed gently and incubated at 37^{o}C in a circulating water bath for 30 minutes. The samples were stored at -80^{o}C after incubation.

Component	Volume (µl) per reaction
10X Calf Intestinal Phosphatase Buffer	0.4
Labelling Spike-In	1.1
Calf Intestinal Phosphatase	0.5
Total	2

Table 3.20: Components of calf intestinal alkaline phosphatase (CIP) master mix

The next day, after thawing the samples, 2.8µl of 100% DMSO was added to each sample and incubated in a circulating water bath at 100^{o}C for 5-10 minutes. After this, samples were immediately transferred on to an ice-water bath. Later, 4.5µl of ligation master mix for T4 ligase (**Table 3.21**) was added and the mixture was incubated at 16^{o}C for 2 hours in a circulating water bath.

Component	Volume (µl) per reaction
10X T4 RNA Ligase Buffer	1
Cyanine3-pCp	3
T4 RNA Ligase	0.5
Total	4.5

Table 3.21: Components of ligation master mix for T4 ligase

3.11.2 Step 2: Drying the samples

Removal of residual DMSO was essential, as it may interfere with the hybridisation reaction. For this, after the 16°C labelling reaction, the samples were dried completely using a vacuum concentrator at 45 to 55°C or on the medium-high heat setting for about 1 hour. To check for sample dryness, the tube was flicked hard to check for pellet strength.

3.11.3 Step 3: Preparation of 10X blocking agent

10X GE blocking agent was mixed with 125µl of nuclease free water and incubated for 4-5 minutes at 37^{o}C. After dissolving the blocking agent completely, the tube was centrifuged at 15,000 g for 5-10s. This was stored at -20^{o}C till further use.

The labelled miRNAs were hybridised with the miRNA probes attached on miRNA microarray slides in next two steps.

3.11.4 Step 4: Preparation for hybridisation of samples

Dried pellets were then re-suspended in 18µl and mixed with the hybridisation mix (**Table 3.22**). The samples were then incubated at 100^{o}C for 5 minutes and placed in ice-water bath for 5 minutes. After a quick centrifugation (10,000g for 20s), samples were loaded on the microarray slides for hybridisation.

Component	Volume (µl) per reaction
Hyb Spike-In	1
10X GE Blocking Agent	4.5
2X Hi-RPM Hybridization Buffer	22.5
Total	28

Table 3.22: Components of hybridisation mix

3.11.5 Step 5: Preparation of the hybridisation assembly

A clean gasket slide (Agilent,) was loaded on to an Agilent SureHyb chamber base with the label facing up so that barcode label was on the left. Samples were loaded from left to right slowly and carefully to avoid the introduction of bubbles. The array slide with the active side down was placed on the SureHyb slide and SureHyb chamber was closed firmly. The mobility of bubbles (if any) was confirmed by vertically rotating the slide before placing the slides into the hybridisation chamber at 55^{o}C with 20rpm rotation for 24 hours. After hybridisation, microarray slides were removed from the SureHyb chambers, labelled, and washed with wash buffer 1 and

2. Dried slides were placed in slide holder covered with aluminium foil and either scanned immediately or stored in an ozone-free chamber for later use.

3.11.6 Scanning and feature extraction from hybridised miRNA microarray slides

The data was extracted using Agilent SureScan scanner and Feature Extraction program (v 10.7.3). Default grid template and miRNA_107_Sept09 protocol was used for this purpose and the scan setting used was described in **Table 3.23**. This program generated a .txt file as result readout.

Scan setting	Values
Scan region	Scan Area (61 x 21.6 mm)
Scan resolution (µM)	5
5 µM scanning mode	Single pass
eXtended Dynamic range	Selected
Dye channel	Green
Green PMT	XDR Hi 100%, XDR Lo 5%

Table 3.23: Scan setting for 8X15K Agilent human miRNA microarray scanning.

3.11.7 miRNA microarray data analysis:

This analysis was performed by Dr. Antti Ylipää, from Tampere University of Technology, Tampere, Finland. The output data was mapped to the latest genome database, zero or negative intensities were replaced with the lowest positive intensity values, the data were quantile normalized, and RMA summarized. Lists were prepared, which compared miRNA expression among various populations and ranked by p-values generated from a paired two-tailed t-test and the Wilcoxon rank sum test.

SECTION I: Genetic regulation of prostate epithelial differentiation

SECTION I: 1. Introduction

According to the recent predictions released by the American Cancer Society, about 240,000 Americans will be newly diagnosed with prostate cancer in 2012 (Siegel et al., 2012). The number of newly diagnosed cancers in the UK in 2010 was about 41,000 (Cancer Research UK report, 2012). These facts establish prostate cancer as the commonest cancer diagnosed in males in these countries. The majority of these cancers are organ confined cancers and can be cured by surgery. The rest are treated with hormone therapy or radiotherapy. Among them, 10-20% of cancers develop a castration-resistant prostate cancer phenotype (CRPC) within 1-3 years of hormonal treatment initiation (Kirby et al., 2011). These patients have a median life expectancy of less than two-years (Saad et al., 2002, Petrylak et al., 2004, Tannock et al., 2004, Carducci et al., 2007, Nilsson et al., 2007, Attard et al., 2009, Sternberg et al., 2009, Kantoff et al., 2010, Ning et al., 2010, Scher et al., 2010, Yap et al., 2011). Several approaches, such as an autologous dendritic cell vaccine Sipuleucel-T and novel androgen signalling blocking agents such as abiraterone acetate and Enzalutamide (MDV3100) are now improving the outlook of CRPC management (Cheever and Higano, 2011, de Bono et al., 2011, Scher et al., 2012a) (**Table I1.1**). However, in spite of these novel and expensive options, the survival of CRPC patients remains very low (Beltran et al., 2011, de Bono et al., 2011, Yap et al., 2011, NICE, 2012). This suggests that, at least in advanced prostate cancers, apart from hormone responsive hyper-proliferation, other targets, such as cancer stem cells (CSCs), must be considered.

Anti-neoplastic drug	Mechanism of action	Median survival
Current options		
Docetaxel (Tannock et al., 2004)	Inhibition of microtubule formation	18.9 months for docetaxel (every 3 weeks) vs. 15.6 months for mitoxantrone
Radium 223 chloride (Parker et al., 2011)	Radiation induced apoptosis	14.0 months for radium-223 vs. 11.2 months for placebo
Cabazitaxel (de Bono et al., 2010)	tubulin-binding taxane drug	15.1 months in cabazitaxel group vs. 12.7 months in the mitoxantrone group
Next Generation therapies		
Abiraterone (de Bono et al., 2011)	Irreversible and selective inhibition of Cyp17	14.8 months in the abiraterone acetate–prednisone group vs. 10.9 months in the placebo–prednisone group
Sipuleucel-T (Kantoff et al., 2010)	Autologous dendritic cell vaccine	25.8 months in the sipuleucel-T group vs. 21.7 months in the placebo group
(MDV3100) Enzalutamide (Scher et al., 2012a)	Androgen receptor antagonist	18.4 months in MDV3100 group vs. 13.6 months on placebo

Table I1.1: Current treatment options for the management of advanced stage prostate cancer. Modified from (Rane et al., 2012).

I1.1 Pathological changes during cancer development:

The CSCs, similar to normal prostate stem cells, exhibit characteristic stem cell attributes, such as self-renewal. However in cancer, the homeostatic balance between processes maintaining hierarchy such as self-renewal, differentiation, and proliferation is lost, resulting in tumour propagation. Progressive telomeric shortening and expression of apoptosis suppressing oncoprotein BCL-2 in luminal

cells in prostatic intraepithelial neoplasia (PIN) suggested that the acquisition of proliferative potential and loss of senescence could be the obvious initial hierarchical perturbations in prostate cancer (Colombel et al., 1993, Meeker et al., 2002). On the other hand, disruptions in regulation of differentiation are subtler in initial stages, where prostate architecture is fairly well maintained. However, these changes gradually become evident in later stages of cancer and certainly in metastatic and castration resistant cancer where basal and luminal cell phenotypes are ill defined (Gleason, 1977). These interruptions in the hierarchical processes are suggested to be primarily responsible for cancer progression, loss of tissue architecture, and altered cellular composition (Collins and Maitland, 2006, Grisanzio and Signoretti, 2008). Therefore, careful consideration of the cellular composition of cancer mass is essential to appreciate differentiation-related perturbations in prostate epithelial hierarchies.

I1.2 Limitations of anti-proliferative/anti-androgenic treatments as a monotherapy

It has been proposed that anti-proliferative/anti-androgenic therapies can remove only differentiated cells and fail to remove undifferentiated cancer cells, such as cancer stem cells (CSCs), which are responsible for subsequent relapse (Hill and Milas, 1989, Liu et al., 2006, Yaromina et al., 2007, Baumann et al., 2008, Maitland and Collins, 2008b, Qin et al., 2012). Targeting undifferentiated cancer cells using differentiation therapy could form such an additional adjuvant approach for better cancer management. Multiple studies now point out that CSCs possess advantageous traits, such as quiescence, immune evasion, enhanced DNA damage response, expression of drug export channels, and an ability to alter their microenvironment, which render them therapy resistant (Frank et al., 2010). As a consequence, unlike their differentiated progeny, CSCs can successfully defend

themselves against conventional anti-neoplastic drugs (Zhou et al., 2009a, Alison et al., 2011). Putting this in the perspective of prostate cancer, treatment of advanced prostate cancer with anti-androgenic drugs would selectively remove only the differentiated luminal cells. As luminal cells form more than 99% of prostate cancer mass, destroying them would reduce tumour size and serum PSA levels significantly. However, it may also lead to rebound enrichment of CSCs, which are non-proliferating androgen independent cells (Gil-Diez de Medina et al., 1998, van Leenders et al., 2001b, Rizzo et al., 2005, Qin et al., 2012). The relative CSC enrichment after chemotherapeutic treatment was also noted in cancers of the breast (Gupta et al., 2009, Hoey et al., 2009), pancreas (Mueller et al., 2009), liver (Ma et al., 2008), lung (Levina et al., 2008), and colon (Dylla et al., 2008). Recent analysis in mouse glioblastoma conclusively demonstrated that CSCs are indeed resistant to conventional therapy and are directly responsible for tumour relapse (Chen et al., 2012a). It is possible that the CSCs in prostate cancer are also responsible for prostate cancer relapse and even for the development of the castration-resistant phenotype. Targeting these CSCs, *along with* their actively proliferating differentiated progeny, is therefore essential for efficient prostate cancer management. One such approach could be the induction of differentiation in resistant CSCs and then targeting the treatment-responsive differentiated progeny by hormone therapy or anti-proliferative drugs.

I1.3 Differentiation therapy

Differentiation therapy is based on the principle that CSCs can be forced towards a terminally differentiated phenotype by the application of intrinsic or extrinsic chemical factors (Pierce and Speers, 1988). Differentiation of CSCs would obliterate the reservoir CSC population, resulting in inhibition of further tumour growth, relapse or metastasis. Pushing CSCs towards a more differentiated phenotype would

increase tumour responsiveness to conventional therapy, as the differentiated cells remain hardwired to undergo cell death have a limited proliferative potential. For example, in prostate, androgen independent CSCs can be forced to differentiate into androgen responsive luminal cells. These luminal cells then can be targeted by drugs that block androgen signalling, or chemo/ radiotherapeutic approaches. Therefore, the use of the differentiation therapy would be synergistic to the pre-existing treatment options (**Figure I1.1**). The combination therapy with these two approaches has a potential to significantly enhance the efficiency of prostate cancer management regimes.

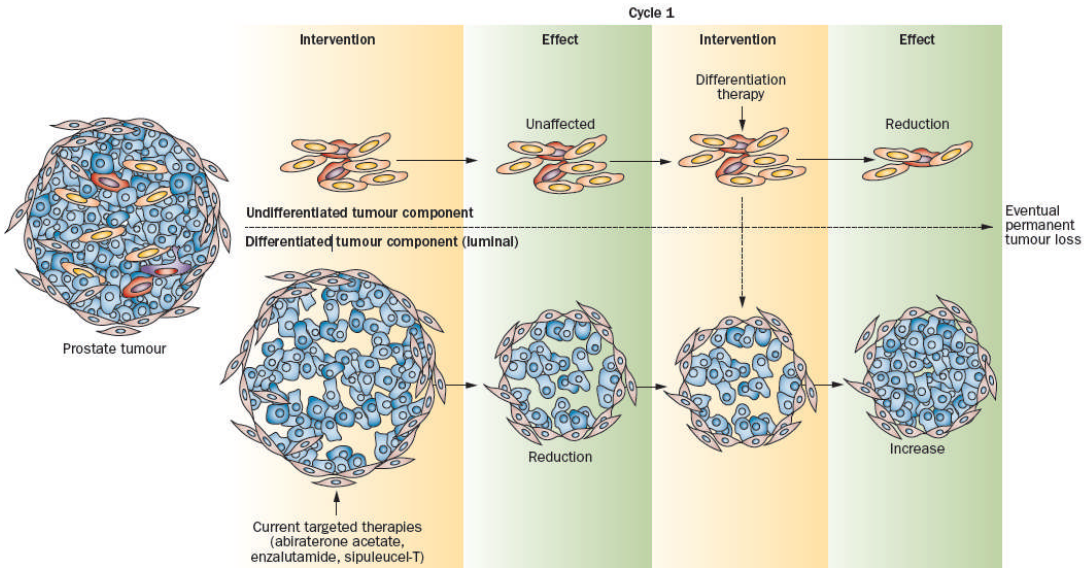


Figure I1.1: Combination therapy (differentiation therapy + current therapies) for efficient prostate cancer management. A cyclical treatment of a prostate cancer with therapy targeting differentiated luminal cells (PSA expressing cells or androgen responsive cells) with subsequent CSC targeting differentiation therapy could prove more beneficial for advanced prostate cancer management. Adapted from (Rane et al., 2012).

I1.4 Differentiation therapy for prostate

I1.4.1 Differentiation therapy for prostate cancer management: Literature review

The conceptually interesting differentiation therapy approach has however failed in pre-clinical assessments in prostate cancer. Limitations in the experimental design and a lack of detailed knowledge about prostate epithelial differentiation have probably caused these failures.

The experimental designs for assessing the pre-clinical and very limited clinical prostate cancer differentiation therapy studies revolved around the evaluation of less than ideal models and experimental end points. The LNCaP cell line was by far the commonest model used for the assessment of drugs that can induce differentiation (Esquenet et al., 1996, Gleave et al., 1998, Hisatake et al., 2000). Since the predominant phenotype in LNCaP cells is a terminally differentiated luminal cells with an less than 1% of relatively undifferentiated cells (Hurt et al., 2008), the effect of any differentiation inducing drug would therefore be insignificant. Ideally, a model cells for differentiation therapy evaluations should contain a significant number of undifferentiated cells. Any reduction in the undifferentiated cell content and increase in the expression of markers of terminally differentiated luminal cells should then be assessed as a treatment end-point. However, with some exceptions (Hedlund et al., 1997, Floryk and Huberman, 2006, Floryk and Thompson, 2008), the majority of studies used ‘decrease’ in PSA (a marker of terminally differentiated luminal cells) as their primary or only evaluation end-point (Debruyne et al., 1998, Denis et al., 1998, Hisatake et al., 2000, Mueller et al., 2000, Woo et al., 2005). During differentiation therapy, PSA expression is in fact expected to rise initially, as induction of differentiation to PSA expressing luminal cells is the prime objective of the therapy. Apart from these pre-clinical studies, the clinical trials

assessing the efficiency of the differentiation therapy also had limited scope. One phase II trial was actually designed for the evaluation of type 2 diabetes mellitus and the assessment of the effect on prostate cancer progression was done retrospectively (Mueller et al., 2000). The second was just a case study (Hisatake et al., 2000), whereas the third one lacked any control arm (Woo et al., 2005). These observations clearly stress the need for a more thoughtful approach to the evaluation of prostate cancer differentiation therapy. It seems that the lack of the precise knowledge about prostate epithelial differentiation has restrained the choice and dose of drugs, hampering the progress of any differentiation therapy.

I1.4.2 The regulation of prostate epithelial differentiation

Relatively few studies have investigated prostate epithelial differentiation at the molecular level. Prostate development begins at the 15.5 days post-coitum (dpc) in mouse, when endodermal outgrowth from the prostatic urethra invades into the FGF10-expressing surrounding mesenchyme, explicitly after stimulation by testosterone (Donjacour et al., 2003, Meeks and Schaeffer, 2010). Distinct duct-like structures are then formed from the endodermal outgrowths, which is at least partly directed by HOX13 paralogs (Economides and Capecchi, 2003). Further development of the duct system is delicately balanced through a positive regulation exerted by Notch, retinoic acid, and PI3-AKT pathways, and negative regulation through WNT, SHH, and BMP pathways (Lamm et al., 2001, Pu et al., 2004, Wang et al., 2006a, Wang et al., 2006b, Allgeier et al., 2008, Vezina et al., 2008). Additional insights into epithelial lineage specification were offered by mouse knock out experiments, which suggested the role of P63 in basal cell specification; whereas NKX3.1, SOX9, FOXA1 were all found to regulate luminal cell differentiation (Bieberich et al., 1996, Gao et al., 2005, Signoretti et al., 2005, Thomsen et al., 2008b). However, these experiments were performed using the developing prostate of rodents as a model, and most of them did not provide a

detailed functional characterisation. The conclusions obtained from these investigations may not be directly applied to adult human prostate epithelium, as the human prostate is functionally and anatomically distinct from the rodent prostate (Shappell et al., 2004). There are extremely limited investigations, which assessed the regulation of normal/cancer '*adult*' human prostate epithelial differentiation. This lack of clear information about adult human prostate epithelial differentiation has therefore resulted in the application of untested differentiation inducing agents with a questionable role in prostate epithelial differentiation in pre-clinical and clinical studies.

I1.4.3 Differentiation inducing agents used in prostate cancer

A wide range of differentiation inducers, such vitamin A and D modulators (Debruyne et al., 1998, Denis et al., 1998, Pasquali et al., 2006, Swami et al., 2011), PPARγ agonists (Kubota et al., 1998), sodium phenylacetate (Samid et al., 1993) and MYCophenolic acid (Floryk and Huberman, 2006), have been evaluated as differentiation inducers in the treatment of prostate cancer. None of these agents have clear or even convincing background data to suggest that they can induce prostate epithelial differentiation. Therefore, not surprisingly, their use as differentiation inducers failed to match with expectations. The risk with the use of these unproven differentiation inducers was illustrated by two recent investigations, which examined the role of PPARγ in prostate cancer management. The first study assumed that PPARγ agonists can induce prostate epithelial differentiation (Leibowitz and Kantoff, 2003), but two recent studies demonstrated that the PPARγ agonist troglitazone induced apoptosis and inhibited proliferation in prostate cancer C4-2 and PC3 cells by altering c-MYC and BCL-xL/BCL-2 signalling via a PPARγ-independent mechanism respectively (Shiau et al., 2005, Akinyeke and Stewart, 2011). There was no evidence for differentiation. A parallel, but more striking example also exists in glioblastoma multiforme (GBM) regarding the use of bone

morphogenic proteins (BMPs) for GBM CSC-targeted differentiation therapy (Piccirillo et al., 2006, Lee et al., 2008a). Piccirillo et al showed that BMPs did induce differentiation in GBM CSCs, and hence, inhibited their tumorigeneic potential (Piccirillo et al., 2006). However, Lee et al. subsequently showed that BMP receptors are epigenetically silenced in the majority of GBM, and the utilisation of BPM agonist in such cases could result in CSC proliferation instead of differentiation (Lee et al., 2008a). This data emphasizes the importance of detailed analysis and functional evaluation of a pathway before its utilisation for the therapeutic purposes. Failure to do so can also result in employment of inappropriate drug doses in pre-clinical studies. For example, compounds such as retinoic acid (RA) analogues were used over a 100,000-fold range, even though it was known that RA analogues have dose-specific effects (Crowe et al., 2003). Studies in last few years have considered some of these issues and have come up with more positive outlooks for prostate cancer differentiation therapy.

I1.4.4 New developments

Three recent investigations provide more direct evidence suggesting that disruption of key molecules/pathways that regulate differentiation could be successfully employed to target prostate CSCs. Two of these studies investigated the interference in the PTEN/PI3K/AKT pathway (Dubrovska et al., 2010) and the CD44 cell surface molecule (Liu et al., 2011). Both of these were previously shown to be critical for the maintenance of prostate stem cells (Charrad et al., 1999, Patrawala et al., 2006, Dubrovska et al., 2009). These studies showed that a significant reduction in the number of $CD44^+$ prostate CSCs, enriched from prostate cancer cell lines, can be achieved by treatment with the PI3K/mTOR modulator NVP-BEZ235 (Dubrovska et al., 2010) and with microRNA miR-34a overexpression (Liu et al., 2011), respectively. More remarkably, a study elucidating the effect of vitamin E derivative γ-tocotrienol on CSCs enriched from PC3 and DU145 cells indicated a

classical response to a differentiation-inducing drug (Luk et al., 2011). The authors of this paper did not stress the role of differentiation, but the results showed typical consequences of induction of CSC differentiation, such as a decrease in the markers of stem cells (CD133 and CD44), prostatosphere formation ability and tumorigenicity in vivo. This result seems somewhat at odds with the outcome of the SELECT trial, where vitamin E increased the total number of *all* tumours, without changing the natural history of the disease (Klein et al., 2011). A discrepancy like this clearly demonstrates the critical need to identify exact doses of selective prostate CSC differentiating agents in order to make differentiation therapy a success.

In our lab, we can enrich pure prostate epithelial sub-populations from patient samples. The stem cells and any of their differentiated progeny can now be specifically probed to investigate the functional effects of any drug and the regulation of the stem cell maintenance can be studied in cells closely resembling to the patient. The microarray expression analysis performed using these cells in 2008 have identified some critical pathways, which could be important for prostate stem cell maintenance, such as the IL-6 and NF-κB pathways (Birnie et al., 2008).

SECTION I: 2. Aims

With the emerging role of cancer stem cells in the progression and relapse of cancer, there is an intense interest in cancer stem cells targeted therapies. The differentiation of treatment-resistant CSCs to treatment sensitive differentiated cells is one of the promising options for targeting CSCs. However, apart from androgen-mediated prostate epithelial differentiation, we do not have a detailed analysis of prostate stem cell differentiation. Therefore, we wanted to investigate the regulation of adult human prostate stem cell differentiation using pure and homogeneous patient-derived prostate epithelial sub-populations. In this analysis, we utilised previously published microarray data (Birnie et al., 2008), assessing the expression profile of stem cells and committed basal cells enriched from BPH and high Gleason-grade treatment naïve prostate cancers (PCa), to identify genes that can regulate prostate cancer stem cell differentiation. Ultimately these pathways (or key nodes in these pathways) can be therapeutically targeted for the development of next generation prostate cancer differentiation therapy.

SECTION I: 3.Results

I3.1 Reanalysis of published microarray data

The published microarray study evaluated the differential gene expression states in pure and homogeneous stem (SC) and committed basal (CB) cell populations isolated from 6 benign and 12 PCa samples (Birnie et al., 2008). The reanalysis of this data was performed because it is suggested that RMA is more specific and sensitive for differential gene expression analysis on microarray dataset comprising of multiple chips thus providing a consistent fold-change across all the microarray chips (Irizarry et al., 2003, Harr and Schlotterer, 2006). Dr. Alastair Droop performed this analysis. A similar trend of RNA degradation plots in all the microarray chips and homogeneous probe level model (PLM) chip images (**Fig. I3.1**) again confirmed the good quality of the raw data. The statistical output of the RMA analyses was a list of 900 genes that are specifically over-expressed in committed basal cells enriched from benign and malignant samples with $p < 0.01$ (by paired two-tailed t-test) (first 50 genes are mentioned in **Appendix 1**), from which candidate genes were chosen. We decided to choose genes that are overexpressed in committed basal cells, instead of genes that are overexpressed in stem cells for three main reasons: (i) Very limited number of genes were up-regulated in stem cells, and although the differences in expression were significant, the magnitude of differences was minimal and (ii) in our experience, it was extremely difficult to perform loss of function studies in stem cells (due to the technical challenges owing to the limited number of stem cells), and (iii) some of the genes over-expressed in stem cells were being investigated in another project.

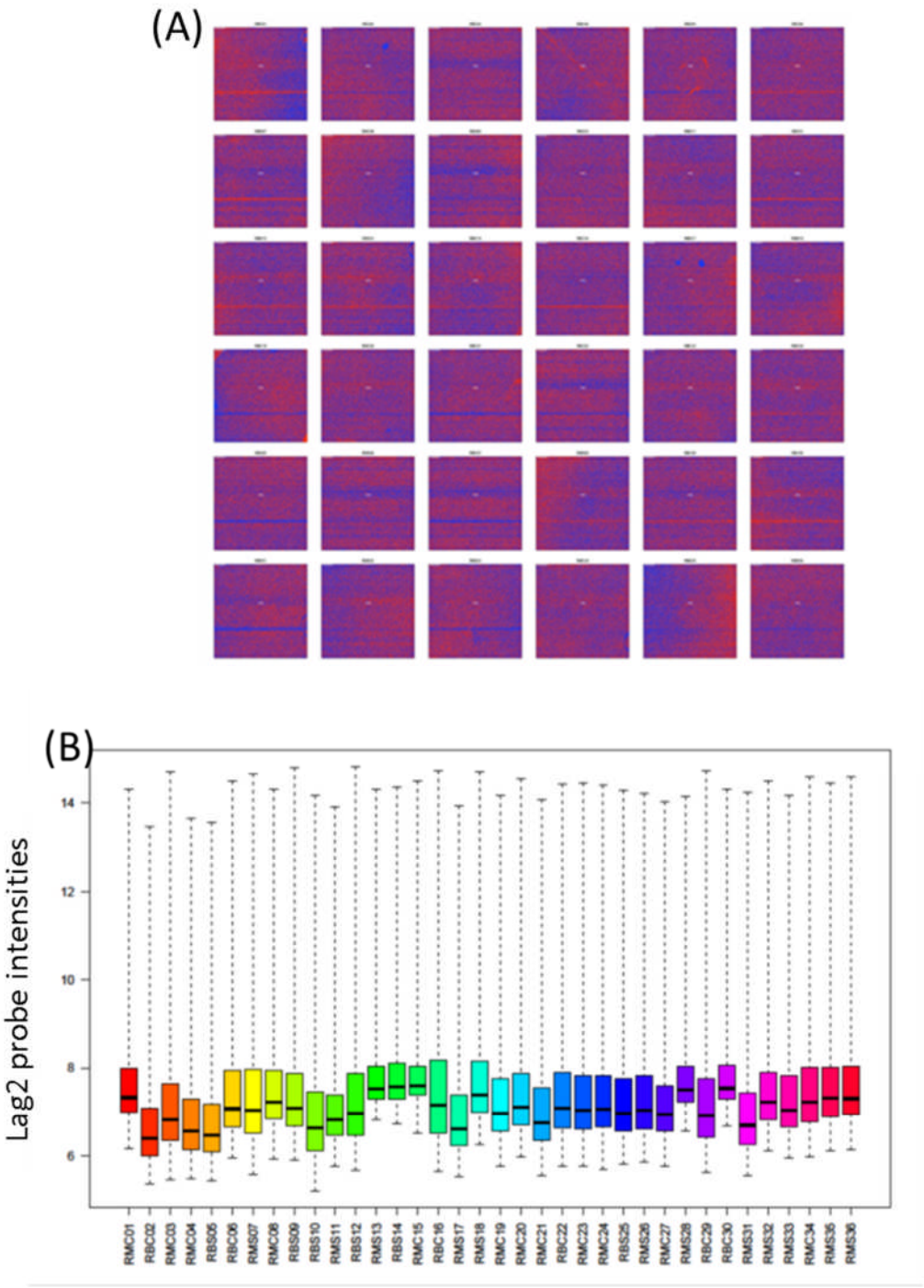


Figure I3.1: Raw microarray data analysis. **A:** Probe level model (PLM) images before normalisation, showing lack of bubbles and large over/under hybridised areas in microarray chips. Each of the small squares is one microarray chip. **B:** Boxplots created by *log*2 transformed probe intensity values that compare the probe intensity levels between the arrays of a dataset, showing that the average hybridisation intensity on each microarray chip is similar. Either end of the box represents the upper and lower quartile. The black line in the middle of the box represents the median. (Diagrams prepared by Dr. Alastair Droop). Small inconsistencies seen here were then eliminated by RMA normalisation.

The next objective was to check if the differentially expressed probes match to any of the known or novel chromosomal changes related to epithelial differentiation. For examples, during epidermal stem cell differentiation, the expression of about 27 genes clustered on 1q21 (EDC cluster) changes during differentiation (Marenholz et al., 2001). In order to check for such changes, the differentially expressed probes between stem cells and committed basal cells in this analysis were mapped to their chromosomal locations by Dr. Alastair Droop (**Figure I3.2**). No specific localisation to any particular chromosomal locus was found. A possible small cluster was detected on chromosome 6p, but it did not correspond to any known differentiation associated co-regulated cluster.

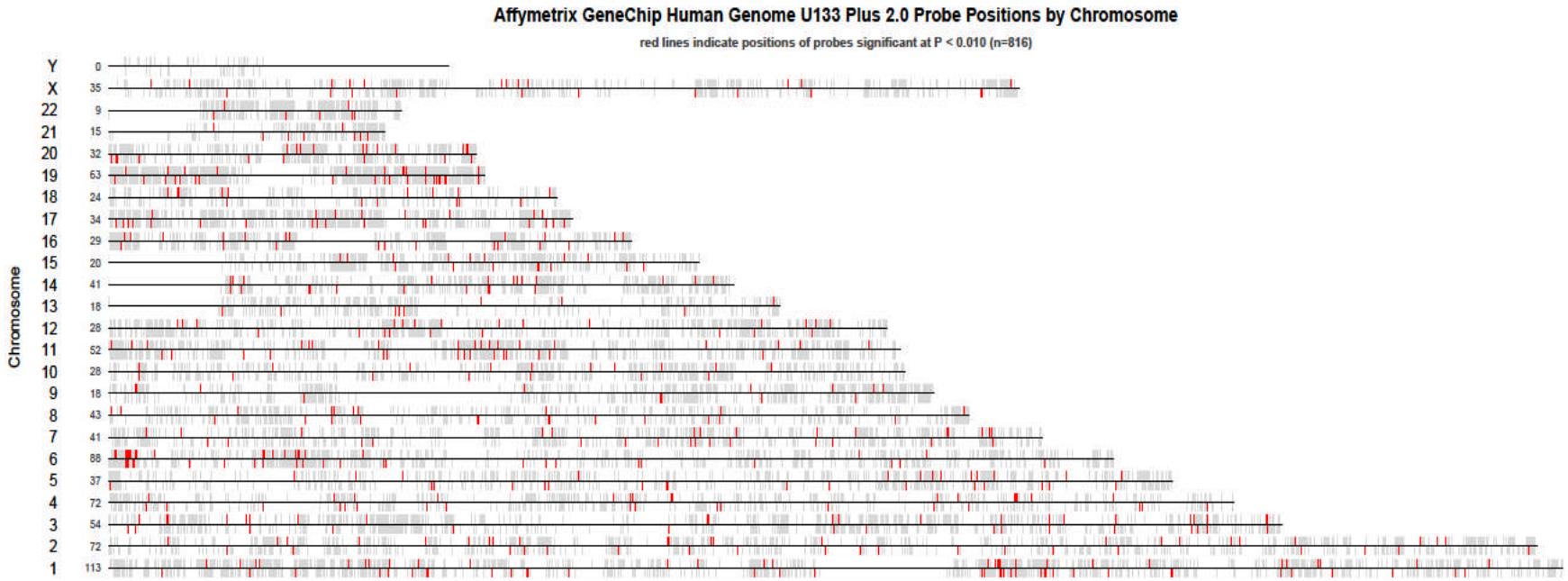


Figure I3.2: Location of differentially expressed genes in stem vs. committed cells (irrespective of pathological origin). The first column of numbers indicates chromosome number and the second column indicates number of differentially expressed genes located on that chromosome with $p<0.01$ by paired two-tailed t-test. Chromosomal length represents their actual length. Genes that are differentially expressed in stem cells are represented by red lines, whereas grey lines represent position of all probes in present on the Affymetrix microarray slide. (Diagram prepared by Dr. Alastair Droop)

I3.2 Selection of candidate genes:

Although we already had some data about the embryonic development of human prostate, the regulators of adult prostate epithelial stem cell fate still remain to be identified. In order to identify these regulators, we picked genes, which were over-expressed in committed basal cells compared stem cells (**Figure I3.3**, **Table I3.1 and I3.2**).

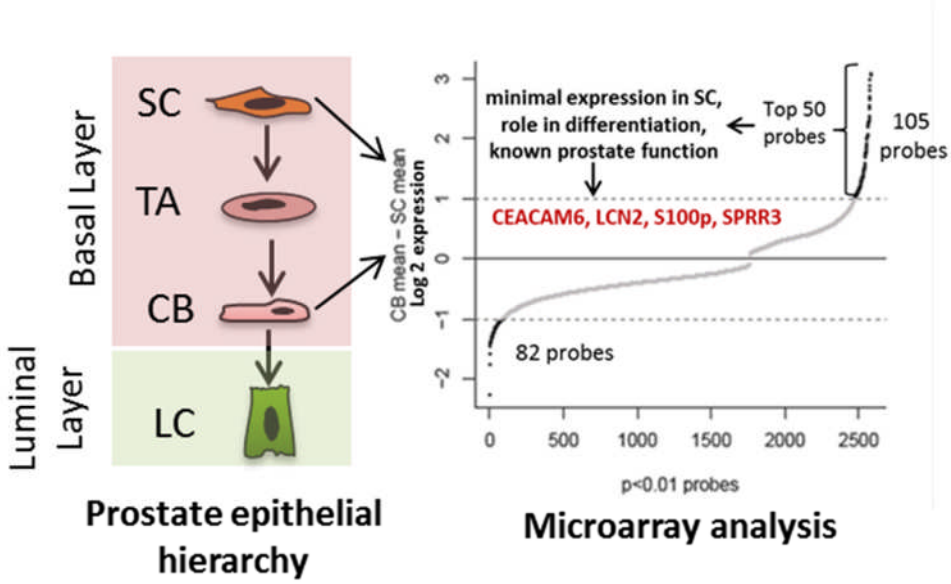


Figure I3.3: **Selection of the candidate genes**. A watershed graph showing probes that were significantly differentially expressed between SC and CB cells. Using the criteria mentioned in the figure, CEACAM6, LCN2, S100p, and SPRR3 were chosen as candidate genes. The graph was made by D. Pellacani.

Gene	Mean expression SC	Mean expression CB	CB/SC	P value
LCN2	9.39	11.18	1.79	0.00026
CEACAM6	6.99	10.07	3.08	0.00001
S100p	8.62	11.18	2.56	0.00007
SPRR3	6.91	9.89	2.98	0.00008

Table I3.1: Relative log2 expression of the candidate genes in the combined microarray data (BPH and PCa). SC: stem cells, CB: committed basal cells. P values are calculated using paired two-tailed t-test.

Gene name	Cellular location	Protein family domain	Functions attributed
LCN2 (24p3, NGAL)	secreted	Binding protein, iron transporter	Role in apoptosis, innate immunity and renal development, NF-kB dependent up-regulation in PCa (Kehrer, 2010, Li and Chan, 2011)
CEACAM6 (NCA, CEAL, CD66c)	Cell membrane	Extracellular domain, Ig-domain, N-terminal domain	Cell-cell signalling, elevated in many solid tumours, including prostate cancer (Blumenthal et al., 2007, Han et al., 2008)
S100p	Cytoplasm, Nucleus	Ca^{++}, Zn^{++}, and Mg^{++} transporter, EF hand	Cell cycle progression and differentiation, Androgen dependent up-regulation in PCa, hypomethylated in PCa (Basu et al., 2008, Jiang et al., 2012)
SPRR3	Cytoplasm	Protein binding, structural constituent of cytoskeleton	Up-regulated during epidermal differentiation and in multiple cancers (Koizumi et al., 1996, Fischer et al., 1999, Kim et al., 2012a)

Table I3.2: Brief information about the candidate genes, which may regulate prostate epithelial differentiation.

I3.3 Candidate gene expression:

I3.3.1 Expression in PPEC derived sub-populations

In spite of quality control on the raw microarray data, sometimes microarray data may not represent the true biological expression pattern. This is due to cross hybridisation of probes or other instrumental/data processing errors. This necessitates direct confirmation of the microarray results. The primary prostate epithelial cells are cultured with irradiated mouse STOs feeder cells, and while selecting SC, TA, and CB cells; STO cells often contaminate CB cell population. Therefore, we first assessed the expression of the candidate genes in STO cells to rule out any significant impact on final analysis due to STO contamination. The expression of all the candidate genes was not detected or detected at

extremely low levels in STOs compared to primary prostate epithelial cultures, suggesting that differential STO contamination in SC, TA, and CB cells won't alter the candidate genes' expression analysis (**Figure I3.4**).

qRT-PCR analysis of candidate genes' expression in sub-populations enriched from BPH (n=6) and treatment naïve cancer (n=6) derived PPECs showed that the mRNAs of LCN2, CEACAM6, S100p, and SPRR3 were significantly over-expressed in CB cells compared to SC and TA cells (**Fig. I3.5-8**). In spite of large inter-sample variations, the expression of the candidate genes in CB cells was significantly different from SC and TA cells in both BPH and PCa. The differential expression was however less marked in sub-populations enriched from castration resistant prostate cancers (CR-PCa, n=2) for all 4 candidate genes. The differential expression was less marked in CR-PCa samples but the trend of expression was similar to that observed in BPH and PCa derived PPECs.

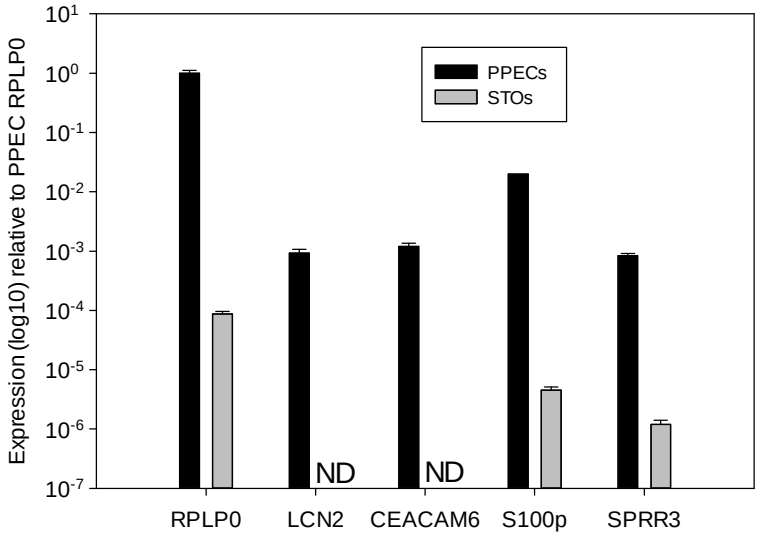


Figure I3.4: qRT-PCR analysis for the expression of the candidate genes and RPLP0 (qRT-PCR internal control) BPH derived primary prostate epithelial cultures (PPECs, n=3) and STOs. ND: not detected

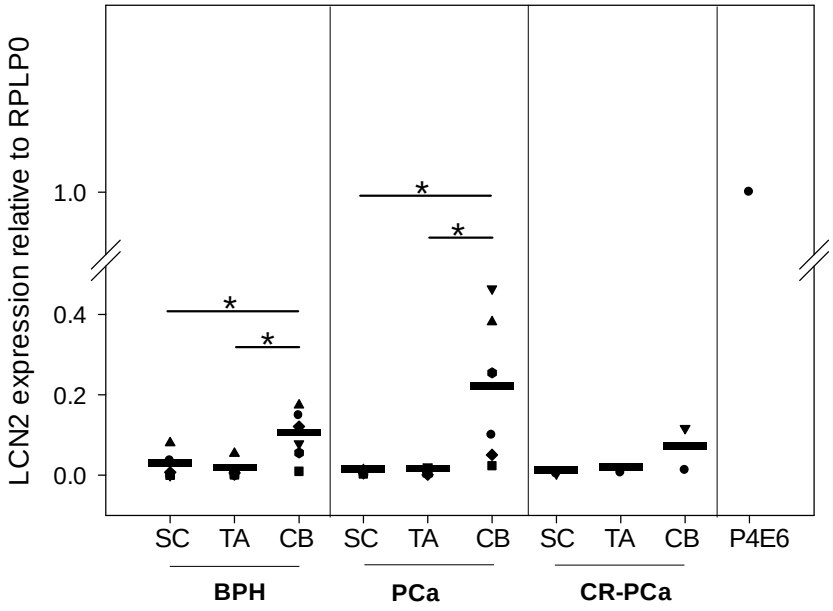


Figure I3.5: Expression of LCN2 mRNA, as analysed by qRT-PCR, in stem cells (SC), transit amplifying cells (TA), and committed basal cells (CB) enriched from benign prostatic hyperplasia (BPH, n=6), treatment naïve prostate cancer (PCa, n=6), and castration resistant prostate cancer (CR-PCa, n=2). RPLP0 was used as an internal control for qRT-PCR and expression was normalised to the expression in prostate cancer P4E6 cell line. Each dot indicates individual patient sample and horizontal line indicates mean. *p<0.05 by paired two-tailed t-test

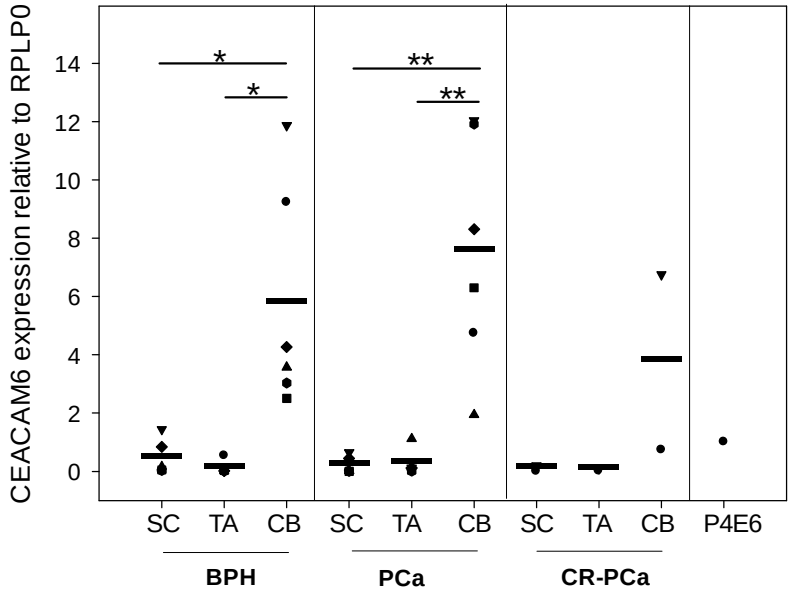


Figure I3.6: Expression of CEACAM6 mRNA, as analysed by qRT-PCR, in stem cells (SC), transit amplifying cells (TA), and committed basal cells (CB) enriched from benign prostatic hyperplasia (BPH, n=6), treatment naïve prostate cancer (PCa, n=6), and castration resistant prostate cancer (CR-PCa, n=2). RPLP0 was used as an internal control for qRT-PCR and expression was normalised to the expression in prostate cancer P4E6 cell line. Each dot indicates individual patient sample and horizontal line indicates mean. *p<0.05 and **p<0.01 by paired two-tailed t-test.

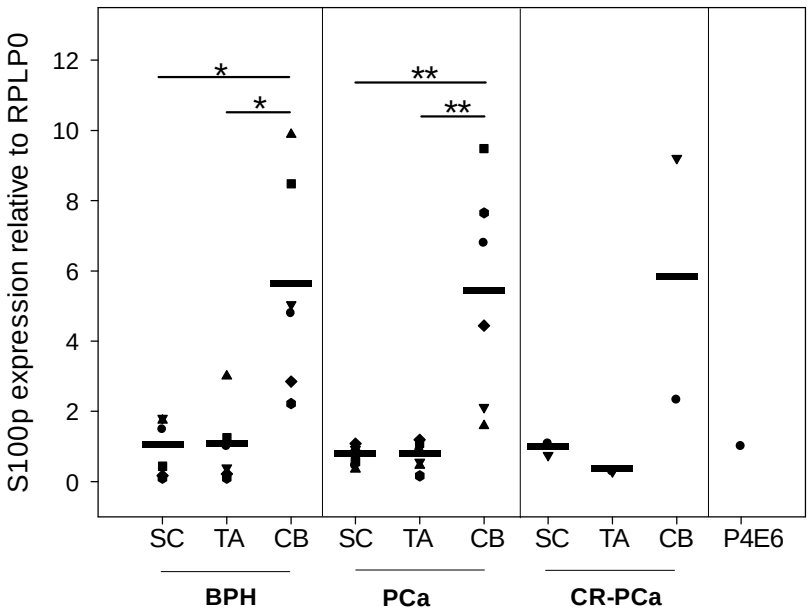


Figure I3.7: Expression of S100p mRNA, as analysed by qRT-PCR, in stem cells (SC), transit amplifying cells (TA), and committed basal cells (CB) enriched from benign prostatic hyperplasia (BPH, n=6), treatment naïve prostate cancer (PCa, n=6), and castration resistant prostate cancer (CR-PCa, n=2). RPLP0 was used as an internal control and expression was normalised to the expression in prostate cancer P4E6 cell line. Each dot indicates individual patient sample and horizontal line indicates mean. *p<0.05 and **p<0.01 by paired two-tailed t-test.

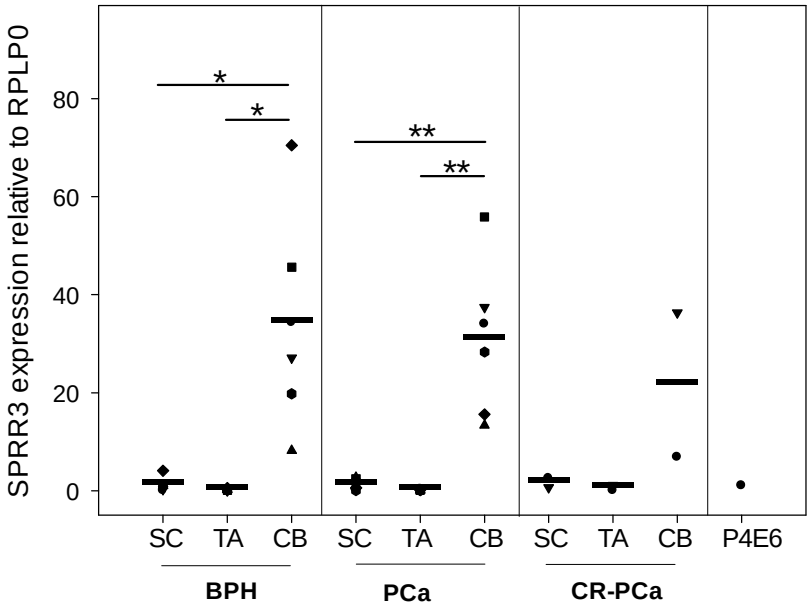


Figure I3.8: Expression of SPRR3 mRNA, as analysed by qRT-PCR, in stem cells (SC), transit amplifying cells (TA), and committed basal cells (CB) enriched from benign prostatic hyperplasia (BPH, n=6), treatment naïve prostate cancer (PCa, n=6), and castration resistant prostate cancer (CR-PCa, n=2). RPLP0 was used as an internal control for qRT-PCR and expression was normalised to the expression in prostate cancer P4E6 cell line. Each dot indicates individual patient sample and horizontal line indicates mean. *p<0.05 and **p<0.01 by paired two-tailed t-test.

I3.3.2 Expression at protein level

The differential mRNA levels were then confirmed at the protein level by western blot analysis. Because of the limited number of stem cells available per sample (~3000/sample), western blot analysis in stem cells could not be carried out. Therefore, the protein expression of the candidate genes was evaluated in TA and CB cells (TA cells being surrogate for SC) (**Figure I3.9**). The overexpression in CB cells was apparent in CB cells derived from BPH and PCa, but not in CR-PCa derived CB cells. S100p was detected at twice the published monomeric molecular weight, suggesting that it may exist as a dimer in primary prostate epithelium. The expression was also compared to the expression in P4E6, PC3, and LNCaP prostate cancer cell lines for future reference (if needed). Expression of all the genes was higher in P4E6, mid in PC3 and lowest in LNCaP cells. Unfortunately, none of the three SPRR3 antibodies tested could detect SPRR3 expression. Since subsequent findings implied that SPRR3 might have a different regulation of gene expression compared to the other three candidate genes, SPRR3 expression analysis was not pursued further.

Together, mRNA and protein data showed that LCN2, CEACAM6, SPRR3, and S100p are overexpressed at mRNA levels in differentiated cells as compared to stem cells, confirming the microarray findings. LCN2, CEACAM6, and possibly S100p are overexpressed in CB cells compared to TA cells at protein levels as well.

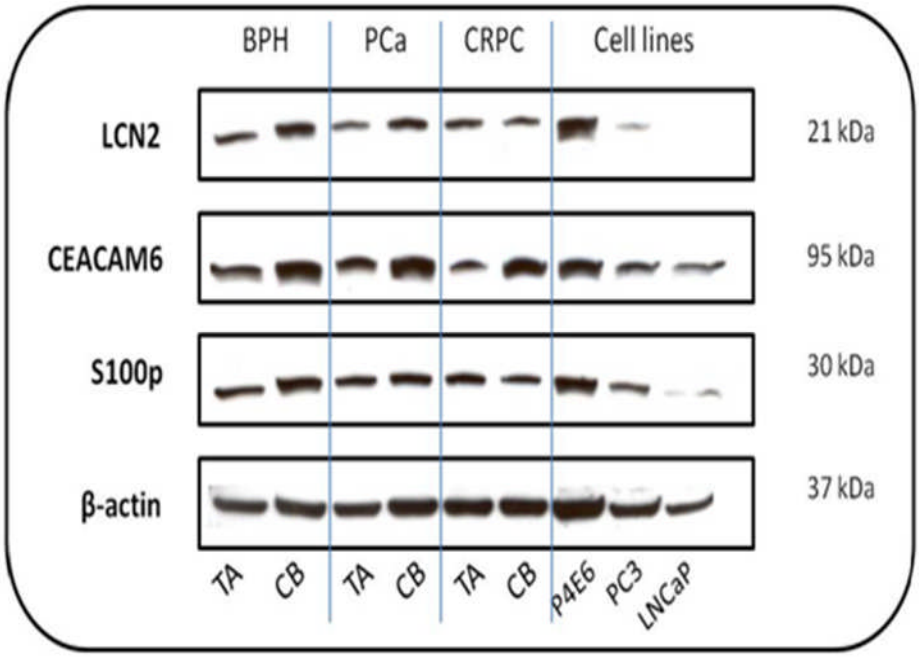


Figure I3.9: Representative image of protein expression of the candidate genes in primary prostate epithelial cultures (PPECs). Western blot analysis was performed on transit amplifying cells (TA) and committed basal cells (CB) from BPH (n=3), PCa (n=3) and CRPC (n=3) derived PPECs and P4E6, PC3, and LNCaP prostate cancer cell lines.

I3.3.3 Expression of the candidate genes in luminal cells

Luminal cells are terminally differentiated prostate epithelial cells. We wanted to investigate if the overexpression of the candidate genes was restricted to the committed basal cells or it was a common feature of all the differentiated progeny. Therefore, luminal cells were enriched from fresh samples before culture and the mRNA levels of the candidate genes was assessed (**Figure I3.10**). The expression of LCN2, CEACAM6, and S100p in luminal cells was similar to that in committed basal cells but the expression of SPRR3 was suppressed by almost 10 fold to that in committed basal cells. This suggests that SPRR3 is involved in the early differentiation process, whereas the other three candidate genes may be necessary for early SC/TA differentiation into committed basal cells and terminal luminal cell differentiation.

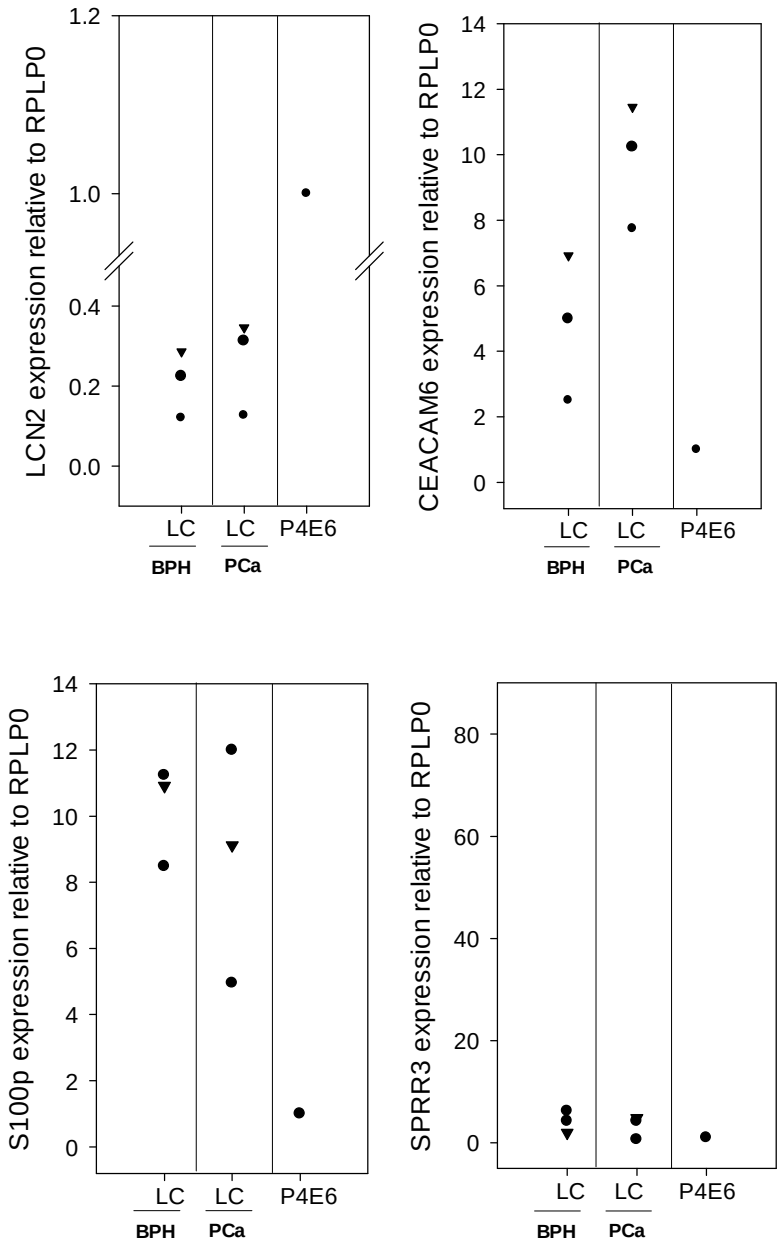


Figure I3.10: Expression of the candidate genes in luminal cells. qRT-PCR analysis was performed on luminal cells enriched from benign prostatic hyperplasia (BPH, n=3) and treatment naïve (PCa, n=3) samples. RPLP0 was used as an internal control for qRT-PCR and expression was normalised to the expression in prostate cancer P4E6 cell line. Each dot indicates individual patient sample. The axes were kept similar to the axes used for that particular gene in previous graphs to aid comparison.

I3.3.4 Expression of the candidate genes in prostate cancer xenografts

Prostate cancer xenografts generated from patient samples provide a good model to investigate the disease progression in vivo. This model may also provide a way to assess the effect of the manipulation of the candidate genes on the cancer stem cell properties and in vivo tumour initiation and progression. Therefore, we assessed the expression of the candidate genes in the sub-populations enriched from prostate cancer xenografts. However, xenografting of prostate tissue into immunocompromised mice provides a completely different environment for cells. This xeno-environment may affect the process of differentiation. When expression of the candidate genes was assessed in the prostate epithelial sub-populations enriched from xenografts, we noticed several changes in the expression pattern compared to the expression in the cultures (**Figure I3.11**). First, expression of all the genes exhibited relatively larger inter-sample variations. Second, the up-regulation in a $CD44^+$ population (equivalent to TA and CB cell combined) was minimal compared to $CD133^+$ (equivalent to SC) population, except for CEACAM6. The average expression of SPRR3 was even higher in the $CD133^+$ population than the $CD44^+$ population. Thirdly, the $CD24^+$ luminal-like cell population exhibited extremely low expression of the candidate genes, except SPRR3. This pattern of expression is completely distorted from what was observed in BPH and treatment naïve cancer-derived PPEC sub-populations, suggesting that normal differentiation (especially luminal differentiation) is disrupted in the xenografts.

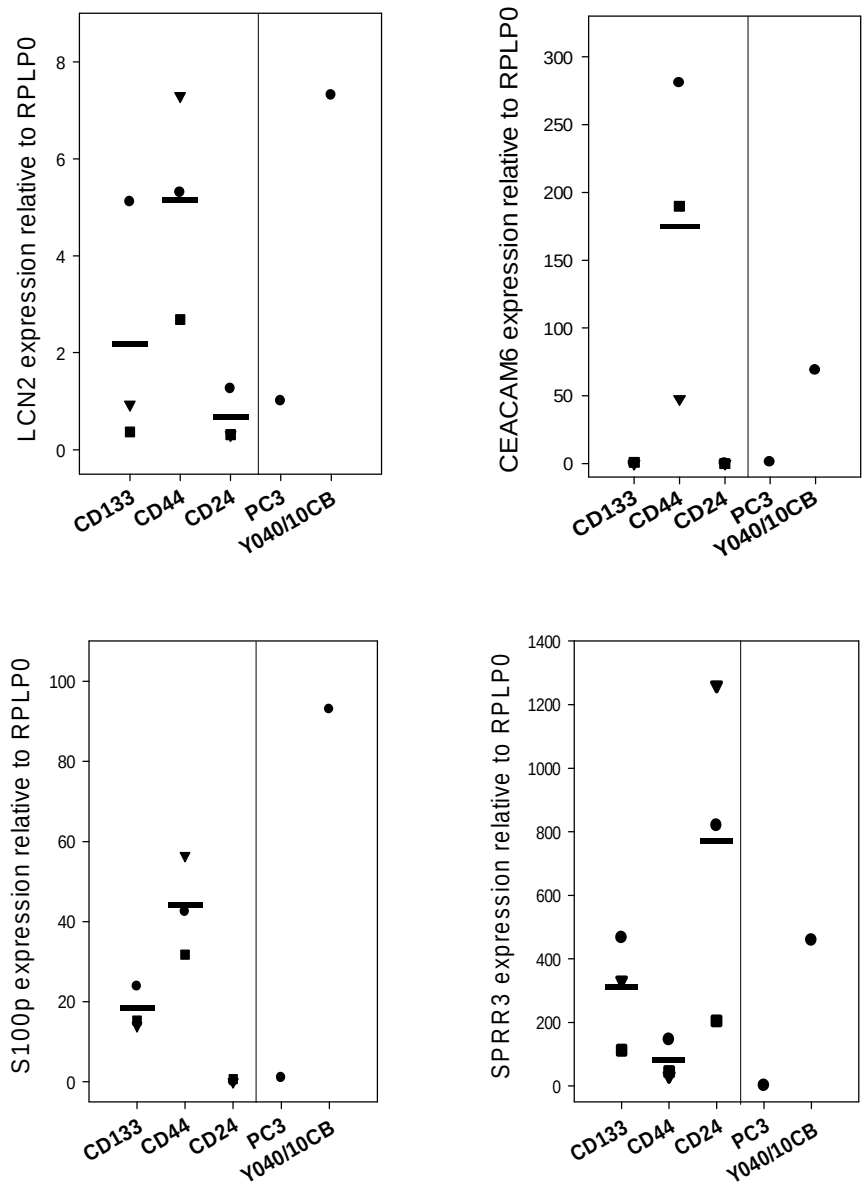


Figure I3.11: Expression of the candidate genes in the prostate epithelial sub-populations enriched from prostate cancer xenografts. qRT-PCR analysis was performed on CD133 (equivalent to SC), CD44 (equivalent to TA and CB cell combined), and CD24 (equivalent to luminal cells) positive cell sub-populations enriched from freshly resected xenografts (n=3). RPLP0 was used as an internal control for qRT-PCR and expression was normalised to the expression in prostate cancer metastatic cell line PC3. The expression of the candidate genes in one of the primary culture derived committed basal cell population was also plotted for comparison. Each dot indicates individual xenograft sample.

I3.3.5 Expression in prostate cell lines

Cell lines provide an additional model to investigate regulatory pathways in depth. They can also be used as a control to compare the newly generated data with previous related data in the same cell line. The data from primary samples suggested that expression of the candidate genes was not significantly different in benign and cancer samples. Expression was also not significantly affected by basal (CB) or luminal (LC) cell phenotype. We wanted to check whether this was also the case with prostate cell lines. Therefore, the expression of the candidate genes was assessed in 7 prostate epithelial cell lines. 2 of these cell lines (PNT1A and PNT2c2) were derived from normal prostate and the rest (P4E6, PC3, LNCaP, VCaP, and PC346c) were prostate cancer-derived cell lines. 4 of the cell lines had a predominantly basal phenotype (PNT1A, PNT2c2, P4E6, and PC3), whereas other 3 (LNCaP, VCaP, and PC346c) had a predominantly luminal phenotype.

There were no obvious differences in benign vs. malignant cell lines, but the cell lines with a basal phenotype had significantly higher expression of the candidate genes than cell lines with a more luminal phenotype (10-1000 fold difference) (**Figure I3.12**). The exception was SPRR3. This pattern was completely different from that observed in the primary samples, where basal and luminal cells had almost similar expression. This suggests that, the basal and luminal phenotypes in cell lines may not represent gene expression status is patient-derived basal and luminal cells in all cases.

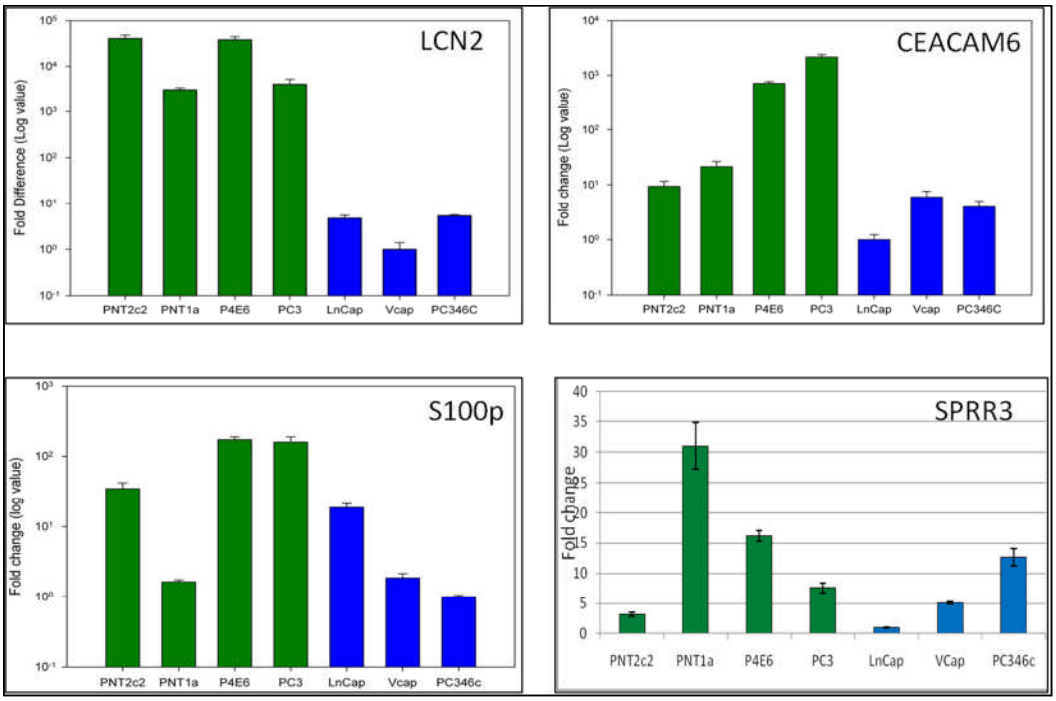


Figure I3.12: Expression of the candidate genes in prostate epithelial cell lines. The expression of LCN2, CEACAM6, S100p, and SPRR3 was assessed by qRT-PCR in prostate epithelial cell lines. Green bars represent cell lines with predominantly basal phenotype and blue bars represent cell line with predominantly luminal phenotype. RPLP0 was used as a qRT-PCR internal control gene. Expression of each gene was normalised to expression in cell line with lowest expression: LCN2 (VCaP), CEACAM6 (LNCaP), S100p (PC346c), and SPRR3 (LNCaP). Experiment done thrice, with three replicates each time. Error bars represent standard error of mean.

I3.4 Expression of candidate genes in other human tissues:

Our analysis showed that the candidate genes were repressed in stem cells and were up-regulated in the differentiated cells. Similar repression of these genes was also seen in other human epithelial tissues. For example, LCN2 and SPRR3 is suppressed in epidermal stem cells and overexpressed in differentiated epidermis (Koizumi et al., 1996, Mallbris et al., 2002). In addition, LCN2, S100p and CEACAM6 were all among the top 25 up-regulated genes after retinoic acid treatment of sebaceous epithelium (Nelson et al., 2008). These findings suggested that the candidate genes may have similar expression patterns across multiple epithelial cells and may respond to certain common transcription factors (such as retinoic acid) in a similar way.

To test this hypothesis, we assessed the expression of the candidate genes in data generated from 806 human microarray experiments. These microarrays represent global gene expression of all the human primary tissues and some cell lines. The microarrays contained cells with normal, malignant, or any other pathological phenotype with and without any drug treatment. In summary, this assembled microarray data provided a snapshot of global gene expression patterns across all the normal and pathological human tissues. This analysis was performed by Dr. Alastair Droop.

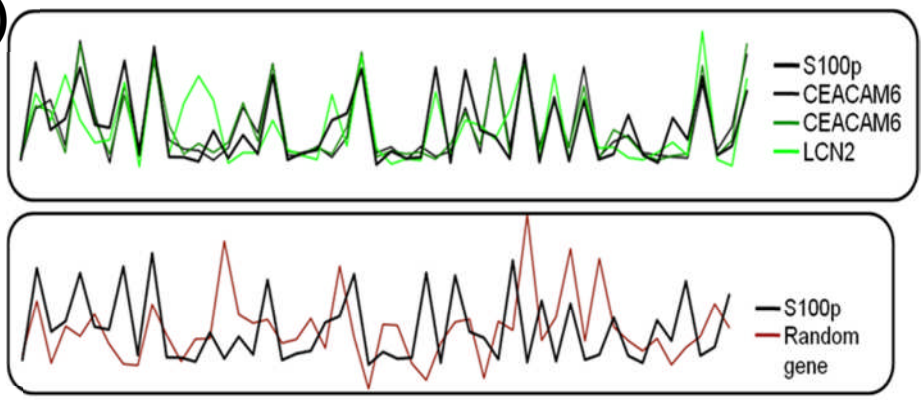
We wanted to assess the expression pattern of our candidate genes across all these microarray experiments and to compare the pattern of expression of one candidate gene with other candidate genes. To our surprise, we found that the expression of S100p was more related to that of CEACAM6 and LCN2 than any other gene in the human genome (**Figure I3.13 and I3.14**). This suggested that LCN2, CEACAM6, and S100p had similar expression patterns in almost all human tissues, and respond to drug treatments in a similar way. Thus, they may

perform similar functions and share common regulatory mechanisms. The fourth candidate gene (SPRR3) did not have a similar expression pattern to the previous 3 genes, but it did have similar expression pattern to genes that form a part of epidermal differentiation cluster along with SPRR3 (**Figure I3.13**). In brief, LCN2, CEACAM6, and S100p could be co-regulated and may have similar functions as they had remarkably similar expression pattern in almost all human tissues.

To confirm this finding in other dataset, we used UCSC genome database. This analysis revealed that LCN2, CEACAM6, and S100p also had very similar expression patterns:

http://genome.ucsc.edu/cgi-bin/hgNear?hgsid=301950779&near_search=uc003gjl.3

(A)



(B)

The top 100 probes correlated to 204351_at

	Probe	cor	Symbol
1	204351_at	1.0000	S100P
2	211657_at	0.6597	CEACAM6
3	203757_s_at	0.6555	CEACAM6
4	212531_at	0.6332	LCN2
5	226226_at	0.5694	TMEM45B
6	205513_at	0.5647	TCN1
7	230323_s_at	0.5647	TMEM45B
8	209173_at	0.5562	AGR2
9	228969_at	0.5508	AGR2
10	201884_at	0.5405	CEACAM5

s_at

Symbol
SPRR3
SPRR3
KRT13
SPRR1A
TMPRSS11D
SPRR1A
SPRR1B
KRT4
CRNN

Figure I3.13: Expression pattern of the candidate genes in the 806 microarray experiments. **A**: Top panel shows the schematic representation of the expression pattern of LCN2, CEACAM6 (2 probes), and S100p at randomly chosen 50 microarray slides from the data analysed. The bottom panel shows the expression pattern of S100p and other randomly chosen gene at the same 50 slides. **B**: List of genes that had similar expression pattern as that of S100p and SPRR3. The blue arrows point at the genes that are involved in epidermal differentiation along with SPRR3.

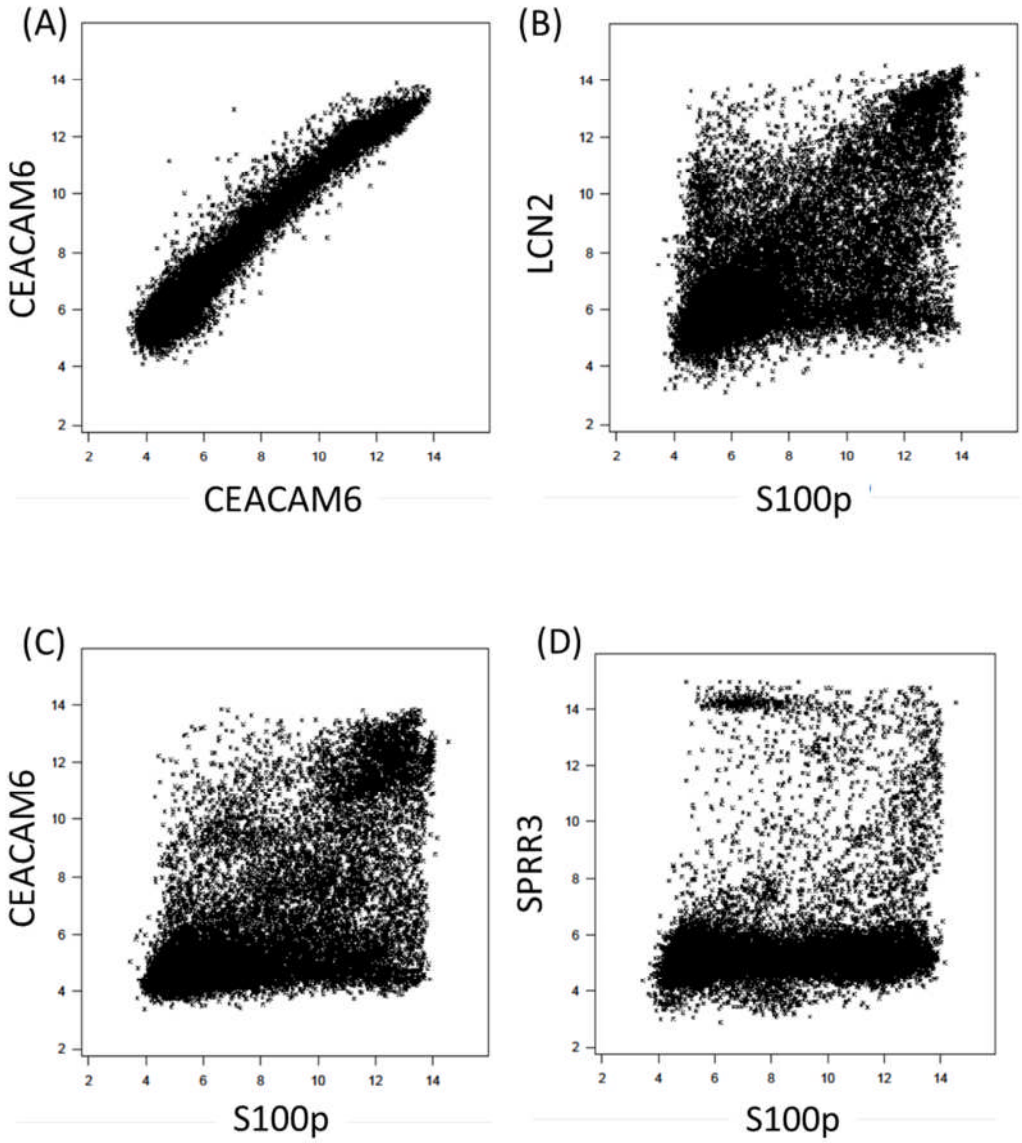


Figure I3.14: Representation of the expression pattern of gene probes in the 806 microarray experiments. Nearly 25,000 slides from 806 human Affymetrix microarrays were assessed for the expression of LCN2, CEACAM6, S100p, and SPRR3. The pattern of expression was plotted in 2D graph, where each axis represents log2 expression of the genes concerned. **A**: Two probes of CEACAM6 plotted against each other with 97% correlation. **B**: Correlation between S100p and LCN2 (63%). **C**: Correlation between S100p and CEACAM6 (67%) **D**: Correlation between S100p and SPRR3 (17%). Diagram prepared by Dr. A. P. Droop

I3.5 Bioinformatic analysis of candidate genes' promoters

Our analysis in prostate and the subsequent analysis of assembled microarray data implied that the candidate genes, especially LCN2, CEACAM6, and S100p could be co-regulated; hence, we decided to assess the promoters of these genes. We hypothesised that if these genes were co-regulated, then they should have binding sites for common transcription factors within the promoter regions.

We found that the promoters of all these genes had binding sites for 8 common transcription factors at their promoters with consensus sequence homology > 80% and $P < 0.001$ and 40 transcription factors had binding sites on the promoters of at least 3 candidate genes with consensus sequence homology > 80% and $P < 0.001$ (**Figure I3.15 and I3.16**).

Some of these transcription factors such as AR, VDR-RXR, SOX9, BCL2, STAT1/3, and NF-κB have been proposed to play a significant role in prostate epithelial differentiation and carcinogenesis (McDonnell et al., 1992, Peehl and Feldman, 2004, Paule et al., 2007, Thomsen et al., 2008a, He and Young, 2009, Rajasekhar et al., 2011). There is a particularly large amount of evidence implying a role for retinoic acid control (Gudas and Wagner, 2011). Therefore, we investigate the potential of retinoic acid signalling in the regulation of expression of candidate genes.

	CEACAM6	LCN2	S100p	SPRR3
AP1		X	X	X
DREAM	X	X	X	
MARE	X	X	X	
PAX3	X	X	X	
SL1		X	X	X
ZNF217	X	X		X
BACH2	X		X	X
GKLF	X	X	X	
MEF3	X	X	X	
PAX6	X	X	X	
SMARCA3		X	X	X
BCL6	X	X	X	
HMX2	X		X	X
MOK2	X	X	X	
PBX1		X	X	X
SOX9		X	X	X
CEBPB		X	X	X
KKLF	X	X	X	
NF-Kb	X	X	X	
RREB1	X	X	X	
TATA	X		X	X

	CEACAM6	LCN2	S100p	SPRR3
HNF4		X	X	X
MZF1	X	X	X	
PEA3	X	X	X	
SP1	X	X	X	
CKROX	X	X	X	
HSF1	X	X	X	
NANOG		X	X	X
PLAG1	X	X		X
SPI1-PU1	X		X	X
DEC--2	X	X	X	
INSM1	X	X	X	
NFAT		X	X	X
PPARG		X	X	X
STAT1	X	X	X	
DINR	X		X	X
IRF2		X	X	X
NFAT5		X	X	X
RFX1	X	X	X	
STAT3	X	X	X	
DMP1	X	X	X	

ARE	BNC	GRE	HBP1	MAZ	OLF1	SRF	TEAD	VDR-RXR

Figure I3.15: Transcription factors that can potentially bind to the promoters of the candidate gens, as determined MatInspector software. Bottom row of 8 transcription factors had binding sites for all the 4 candidate genes. X indicates the presence of at least one binding site for the transcription factor on the promoter.

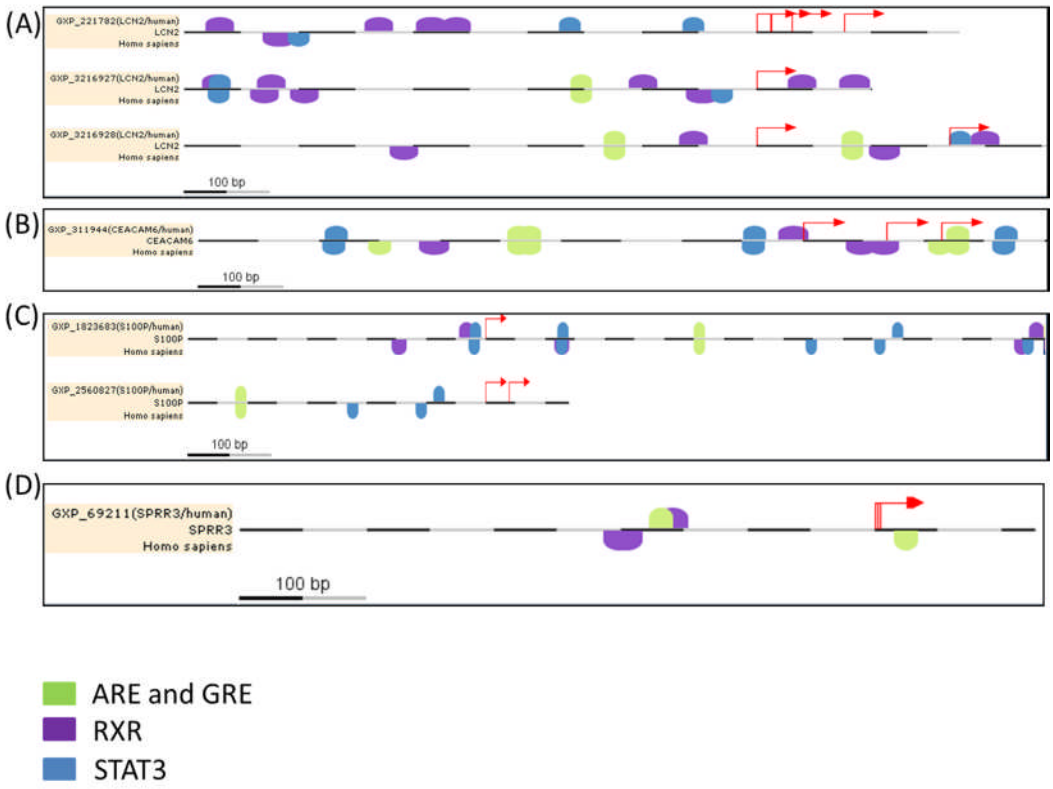


Figure I3.16: The location of androgen responsive elements (ARE), glucocorticoid responsive elements (GRE), Retinoid X receptor (RXR), and STAT3 on the promoters of the candidate genes, as predicted by MatInspector software. A: Three splice variants of LCN2 showing binding sites for ARE/GRE, RXR, and STAT3. B: CEACAM6 promoter C: S100p splice variants D: SPRR3 promoter. Note that SPRR3 promoter does not have a binding site for STAT3. Red arrows indicate transcription start sites.

I3.6 Retinoic acid mediated regulation of candidate genes

Retinoic acid (RA) is a metabolite of vitamin A. It was proposed more than 2 decades ago that the retinoic acid receptor-mediated transcriptional regulation could be instrumental in promoting prostate epithelial differentiation (Peehl et al., 1993). Retinoic acid acts through two types of receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each of these receptors had 3 isotypes: α, β, and γ. RARα is also referred to as RARA and RARβ as RARB etc. In order to identify whether RA regulates the expression of candidate genes, we first determined the expression status of these receptors in prostate cell lines, and primary prostate epithelial cultures.

I3.6.1 Expression of retinoic acid receptors in prostate cell lines and primary prostate epithelial cultures

All the prostate cancer cell lines (benign, malignant, metastatic cell lines) exhibit similar RA receptor expression patterns (**Figure I3.17A**). RXRA had the highest expression in all the cell lines compared to all other RA receptor sub-types, whereas RXRB and RXRG had insignificant expression. On average, the expression of RA receptors was lower in cancer cell lines (P4E6, PC3, LNCaP, VCaP, and DU145) compared to benign/normal cell lines (PNT2c2, PNT1a, and BPH1). No such difference in the primary benign and malignant prostate epithelial cultures was however observed (**Figure I3.17B**). RARG and RXRA had higher expression in primary cultures compared to the other receptors. The expression of RXRB and RXRG was also insignificant in the primary cultures. The expression of RA receptors was also present at the protein level in prostate cell lines and in primary cultures (Rivera-Gonzalez et al., 2012) and (E. Oldridge PhD thesis 2012).These data imply that RA-mediated signalling could be

predominantly mediated by RXRA in prostate cell lines and in primary cultures, although RARA and RARG could also have functional roles.

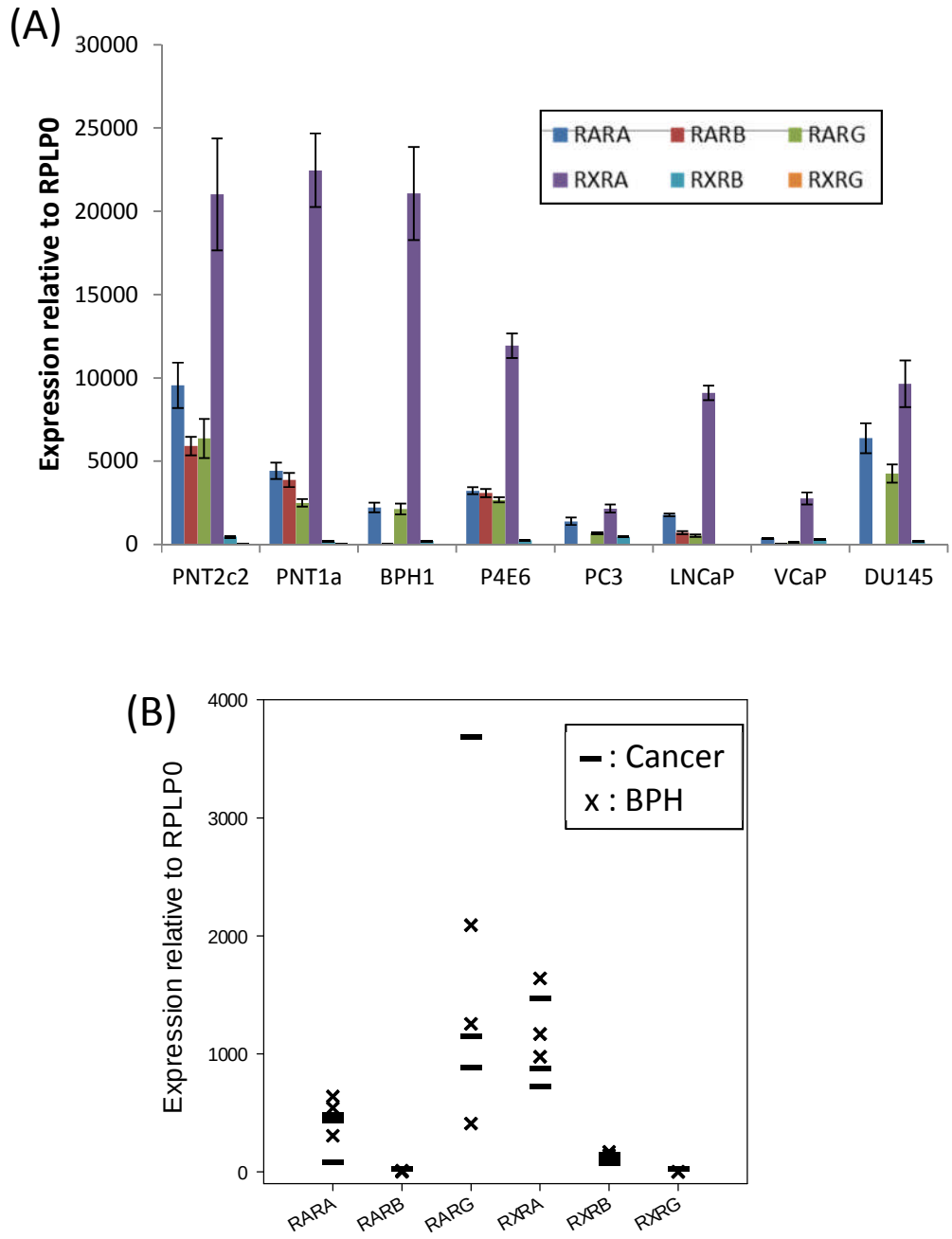


Figure I3.17: Expression of retinoic acid (RA) receptors at mRNA level in prostate epithelial cell lines and primary prostate epithelial cultures. **A**: qRT-PCR analysis for RA receptors in benign/normal cell lines (PNT2c2, PNT1a, and BPH1) and in malignant cell lines (P4E6, PC3, LNCaP, VCaP, and DU145). **B**: qRT-PCR analysis for RA receptors in primary prostate epithelial cultures derived from benign (n=3) and treatment naïve malignant (n=3) samples.

I3.6.2 Effect of stimulation of retinoic acid receptors on the expression of candidate genes

I3.6.2.1 Cell lines:

Once we had confirmed that retinoic acid receptors are expressed in prostate cell lines and primary prostate epithelial cultures, we investigated the effect of stimulation of RA receptors by all trans-retinoic acid (ATRA). RA receptors exist as homodimers or heterodimers. Homodimers are more common with RARs, whereas RXR form heterodimers with several other related receptors, more often with one of the RARs or with the vitamin D receptor (VDR) (Lefebvre et al., 2010). ATRA is an agonist for both of these receptors (RARs and RXRs).

We first investigated the effect of ATRA treatment on candidate genes' expression using prostate cell lines. Normal (PNT2c2) and malignant (LnCaP and PC3) cell lines were treated with ATRA at three different concentrations (10nM, 100nM, and 1000nM) for up to 7 days. A relatively modest but significant (2-5 fold) (with paired two-tailed t-test) dose dependent up-regulation of LCN2, CEACAM6, and S100p was observed; whereas SPRR3 expression was significantly downregulated over 7 days in all 3 cell lines (**Figure I3.18-20**). The pattern of up-regulation of LCN2, CEACAM6, and S100p was different: LCN2 showed gradual up-regulation over the period of 7 days, CEACAM6 showed peak up-regulation after 3 days, and significant up-regulation in S100p expression was observed only after 7 days of treatment. None of these three genes was up-regulated significantly within 1 day after treatment, indicating that ATRA mediated gene activation may not be a direct activation. SPRR3 expression was however significantly downregulated even after 1 day of ATRA treatment, suggesting that it ATRA can supress SPRR3 expression directly. No

obvious morphological changes were observed in any of the cell lines, at any time of treatment.

These results supported the findings of the bioinformatic analysis, which discovered that only LCN2, CEACAM6, and S100p had a similar expression pattern among the candidate genes (**Figure I3.13**).

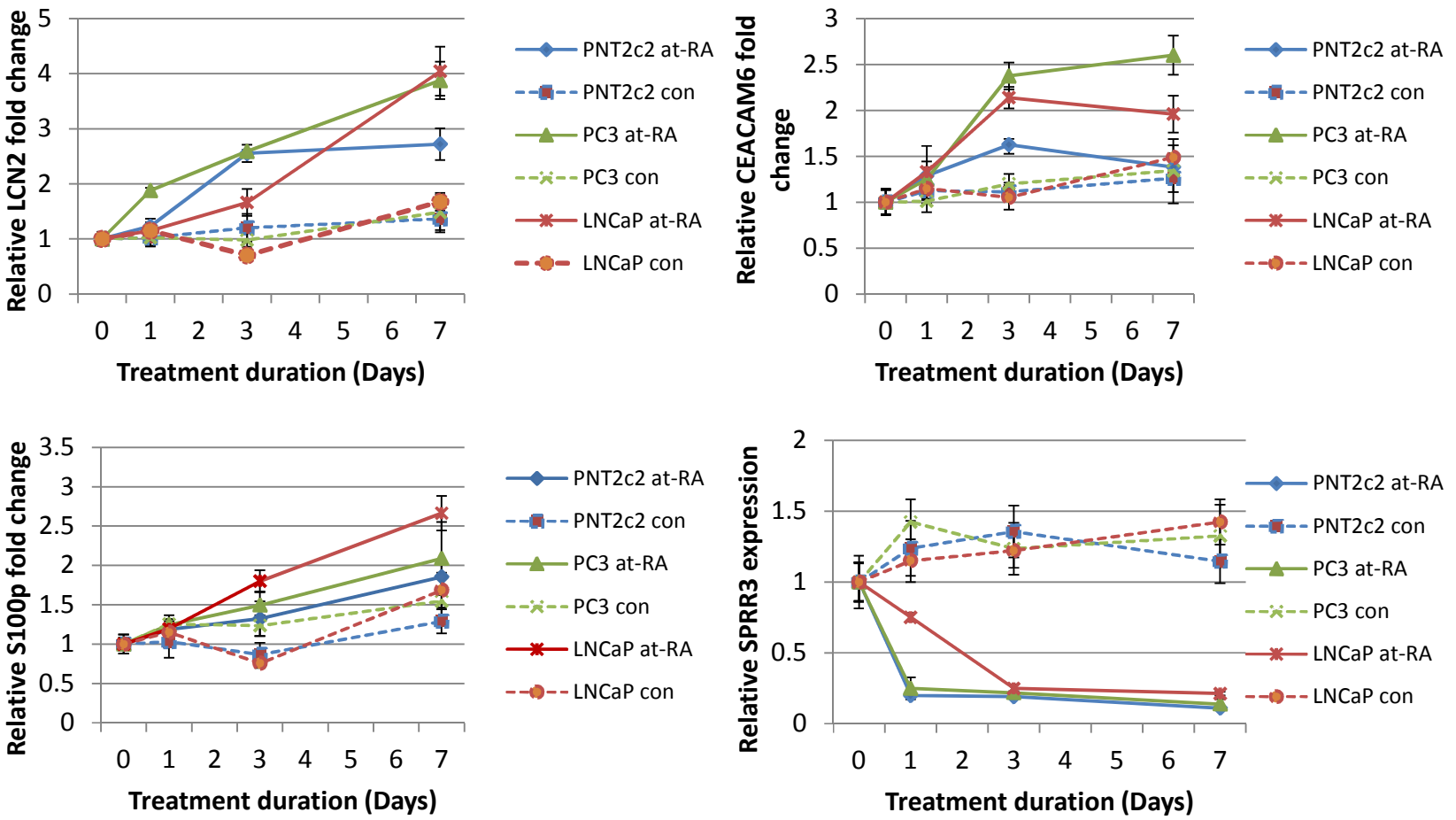


Figure I3.18: Treatment of PNT2C2, PC3 and LNCaP prostate cell lines with 10nM all-trans retinoic acid (at-RA). Three prostate cell lines were treated with 10nM ATRA or an equimolar ethanol control for up to 7 days. New drug in fresh media was added every day. The expression of LCN2, CEACAM6, S100p, and SPRR3 was measured before the start of experiment (time 0) and after 1, 3, and 7 days of treatment initiation by qRT-PCR. RPLP0 was used an internal qRT-PCR control.

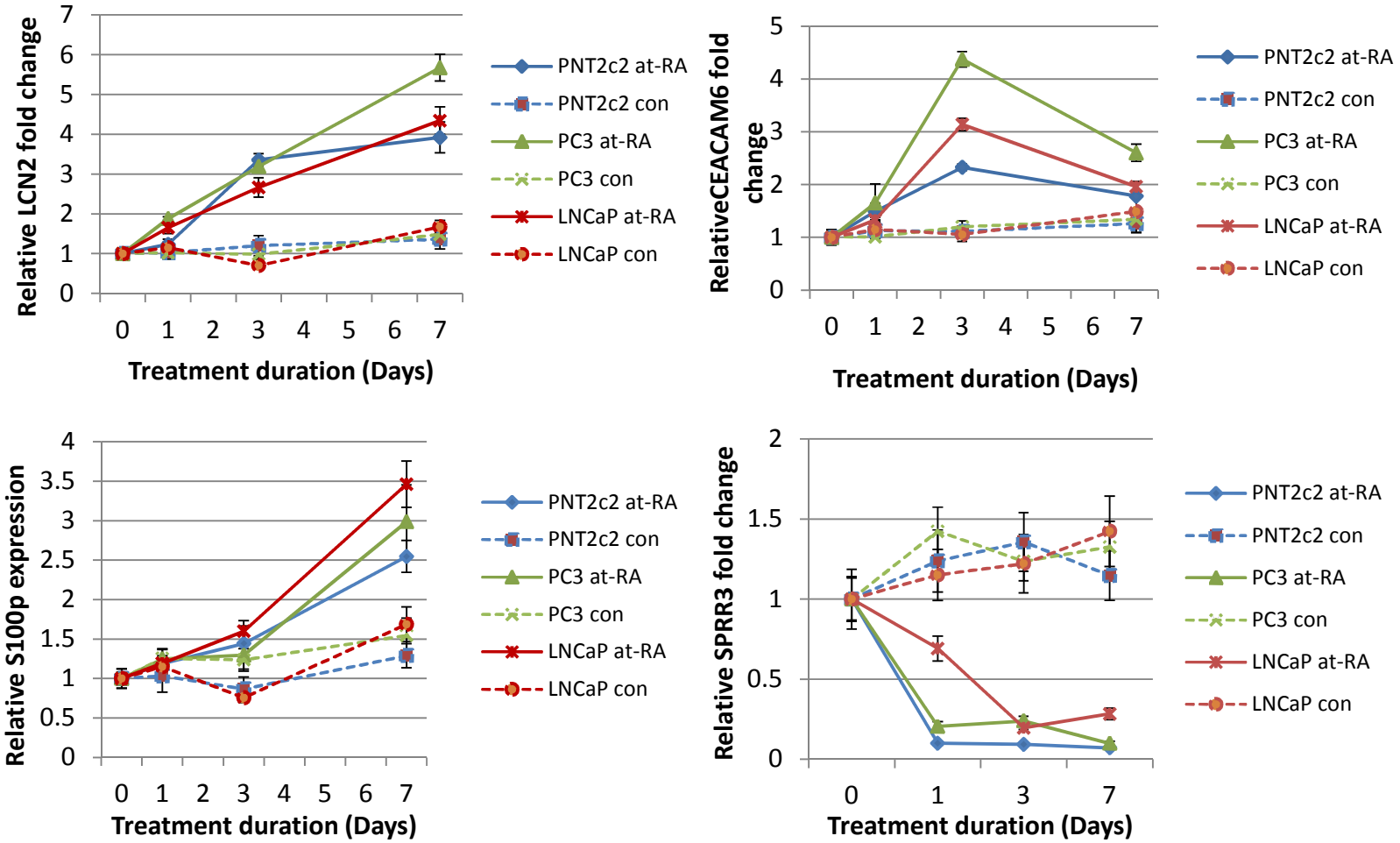


Figure I3.19: Treatment of PNT2C2, PC3 and LNCaP prostate cell lines with 100nM all-trans retinoic acid (at-RA). Three prostate cell lines were treated with 100nM ATRA or an equimolar ethanol control for up to 7 days. New drug in fresh media was added every day. The expression of LCN2, CEACAM6, S100p, and SPRR3 was measured before the start of experiment (time 0) and after 1, 3, and 7 days of treatment initiation by qRT-PCR. RPLP0 was used an internal qRT-PCR control.

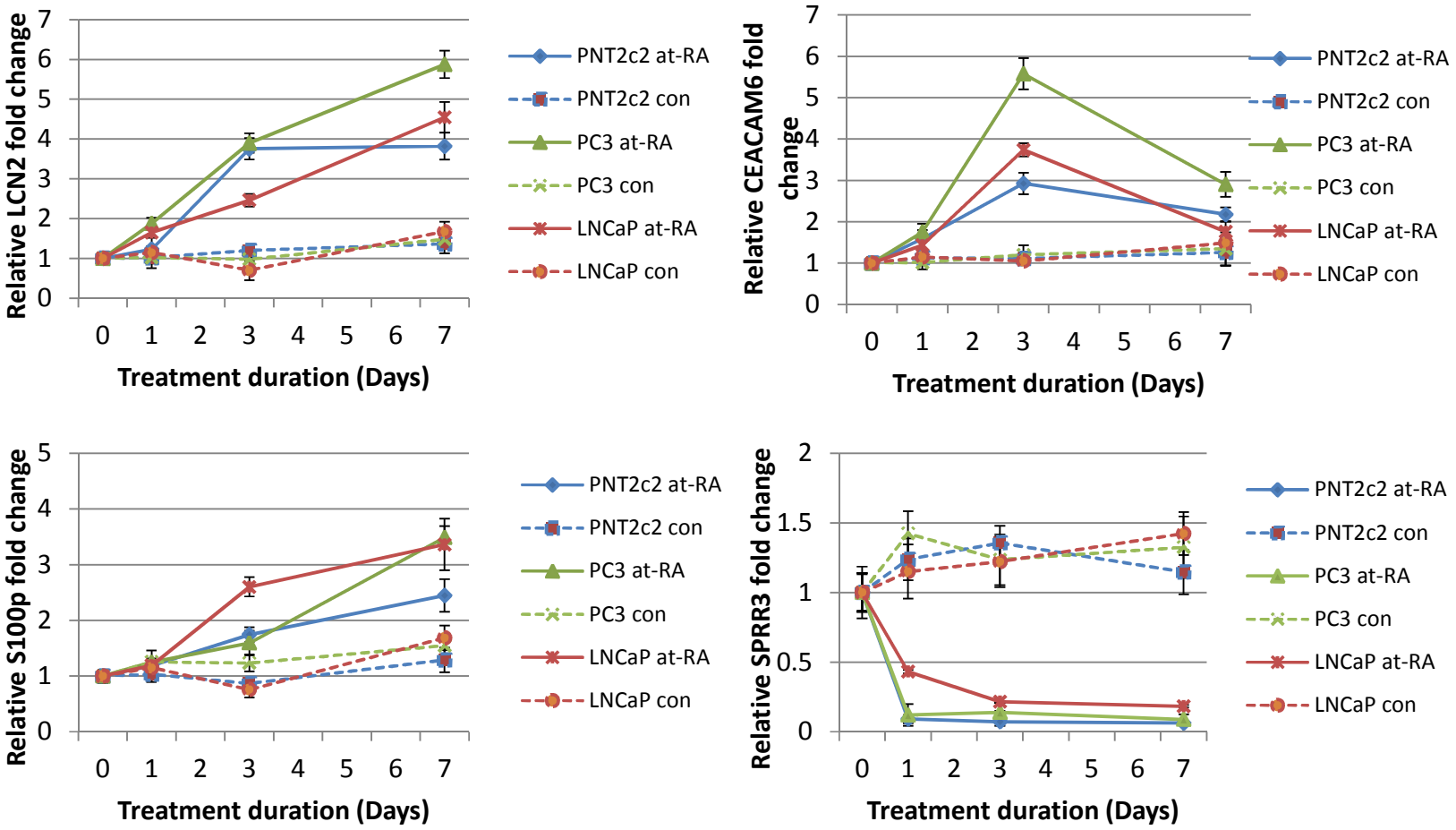


Figure I3.20: Treatment of PNT2C2, PC3 and LNCaP prostate cell lines with 1000nM all-trans retinoic acid (at-RA). Three prostate cell lines were treated with 1000nM ATRA or an equimolar ethanol control for up to 7 days. New drug in fresh media was added every day. The expression of LCN2, CEACAM6, S100p, and SPRR3 was measured before the start of experiment (time 0) and after 1, 3, and 7 days of treatment initiation by qRT-PCR. RPLP0 was used an internal qRT-PCR control.

I3.6.2.2 Primary prostate epithelial cultures:

Based on the cell line data, previously published work and work performed by E. Oldridge in our lab, we used a 100nM concentration of ATRA to assess RA-mediated regulation of the candidate genes in primary prostate epithelial cultures (PPECs). In addition to ATRA treatment, PPECs were also treated with a combination of 9-cis-RA and 1α, 25-dihydroxyvitamin D_3. As the promoters of the candidate genes had binding sites for VDR-RXR receptors, the combination of 9-cis-RA and 1α, 25-dihydroxyvitamin D_3 could specifically activate VDR-RXR receptor and would be more potent than ATRA. The dose of 9-cis-RA (100nM) and 1α, 25-dihydroxyvitamin D_3 (10nM) was selected based on the literature analysis (Brown et al., 1994, Zhao et al., 1997). The pattern of up-regulation of expression of the candidate genes in PPECs remained similar to that observed in cell lines with ATRA and also with a combination of 9-cis-RA and 1α, 25-dihydroxyvitamin D_3, but the magnitude of change was larger (5-25 in PPECs fold vs. 2-5 fold in cell lines) (**Figure I3.21**). Up-regulation of LCN2, CEACAM6, and S100p was observed as in cell lines but the induction was not immediate. In contrast, expression of SPRR3 was downregulated significantly, even after only 24 hours of treatment. When the PPECs were fractionated into SC, TA, and CB sub-populations, the same pattern of change in the expression of the candidate genes was observed, although the overexpression of S100p failed to reach statistical significance (**Figure I3.22**). Similar to cell lines, no obvious morphological changes were observed in any sample, at any time of treatment.

In summary, retinoic acid receptor agonists can up-regulate the expression of LCN2, CEACAM6, and S100p, and supress the expression of SPRR3; without causing any overt morphological changes in cell phenotype. As the SPRR3 expression pattern was different from other candidate genes, we did not investigate SPRR3 expression in our next experiment.

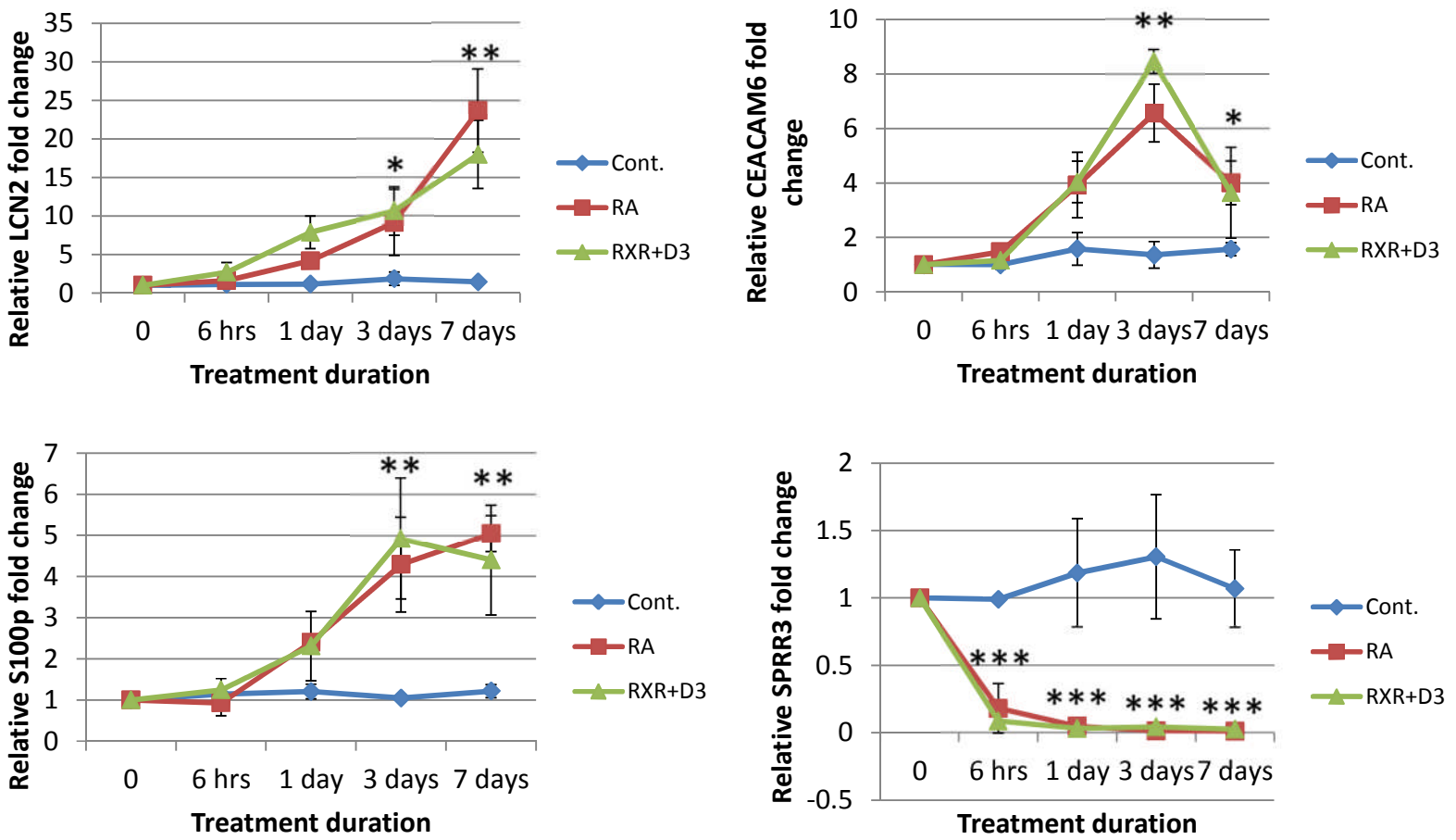


Figure I3.21: Treatment of primary prostate epithelial cultures (PPECs) with all-trans retinoic acid (at-RA) and a 9-cis-RA and 1α, 25-dihydroxyvitamin D_3 (RXR+D3) combination. Three BPH-derived PPECs were treated with 100nM ATRA or 100nMRXR+10nM D3 or an equimolar ethanol control for up to 7 days. New drug in fresh media was added every day. The expression of LCN2, CEACAM6, S100p, and SPRR3 was measured before the start of experiment (time 0) and after 6 hours, 1, 3, and 7 days of treatment initiation by qRT-PCR. RPLP0 was used an internal qRT-PCR control. *p<0.05, **p<0.01, ***p<0.001 by paired two-tailed t-test.

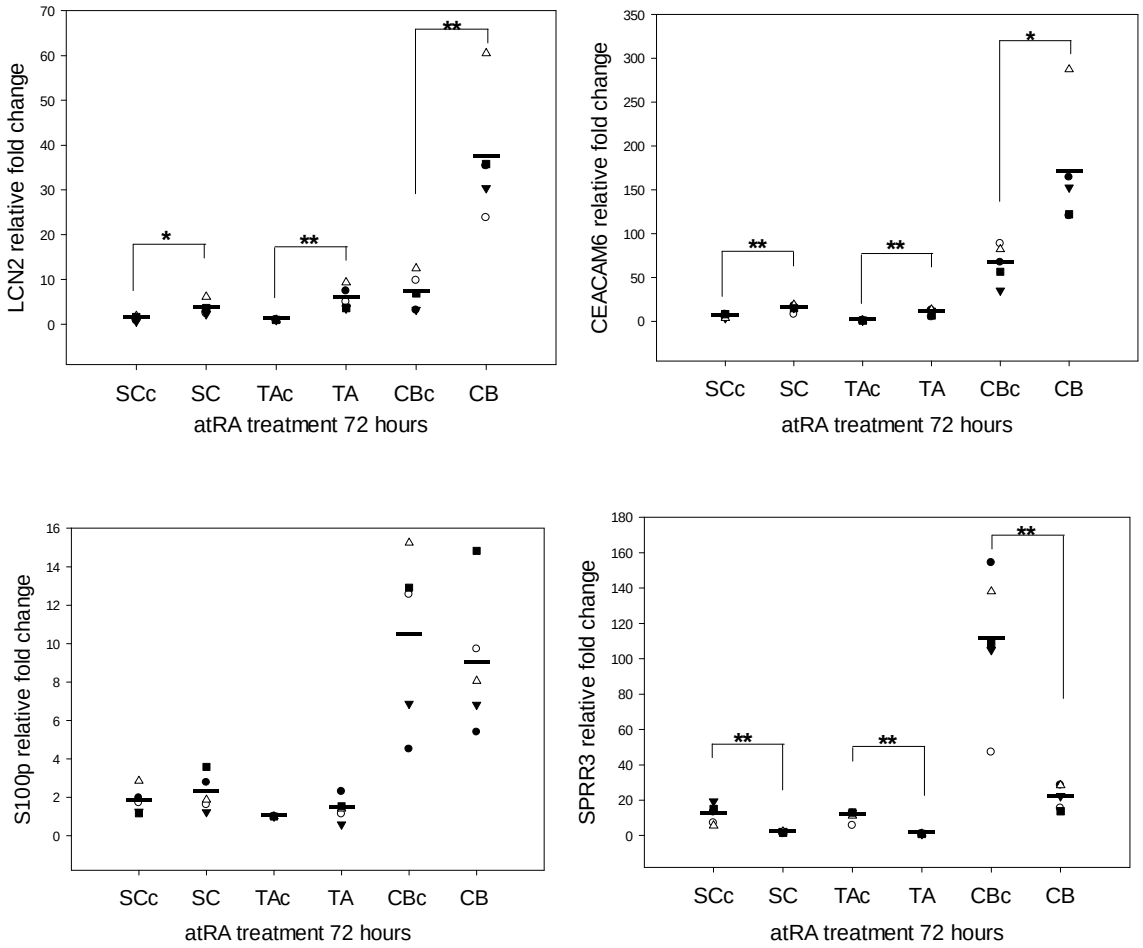
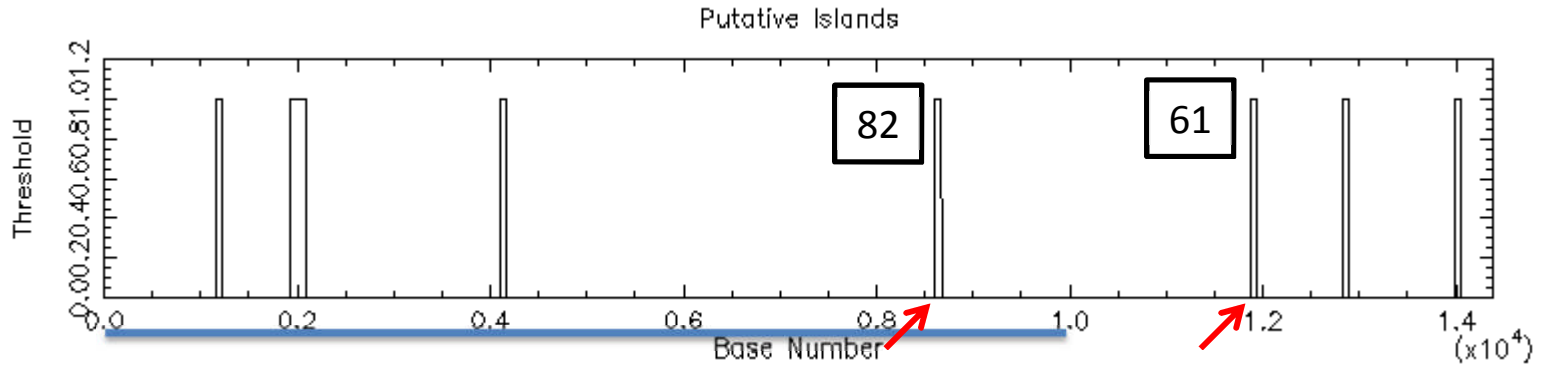


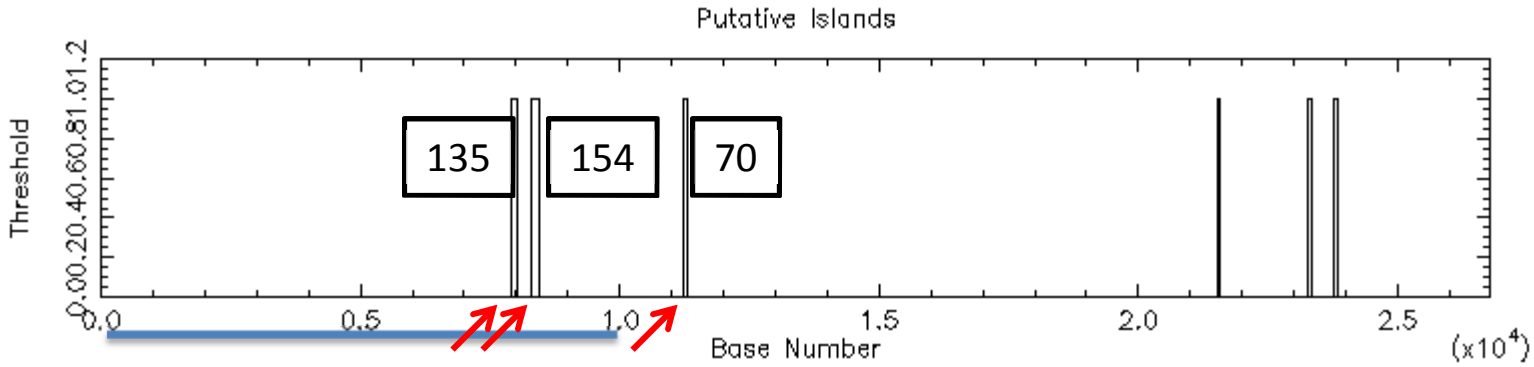
Figure I3.22: Treatment of primary prostate epithelial cultures (PPECs) with all-trans retinoic acid (at-RA). Three BPH-derived PPECs (filled symbols) and 2 cancer-derived PPECs (hollow symbols) were treated with 100nM ATRA or an equimolar ethanol control for 3 days. New drug in fresh media was added every day. Sub-populations were enriched after treatment and the expression was by qRT-PCR. RPLP0 was used an internal qRT-PCR control. *p<0.05 and **p<0.01 by paired two-tailed t-test.

I3.7 Epigenetic regulation of the expression of the candidate genes

Epigenetic mechanisms often work on a genome-wide scale, influencing expression of several genes at a time. Therefore, we decided to investigate the possible role of DNA methylation and histone acetylation on the expression of the candidate genes. First, we located CpG islands at the promoters of candidate genes using EMBL-EBI EMBOSS cpgplot software (Rice et al., 2000). This analysis revealed that the promoters of LCN2, CEACAM6, and S100p do possess CpG islands, but they were not as large as CpG islands seen in classical DNA methylation regulated genes, such as GSTP1 (Millar et al., 1999) (**Figure I3.23 and I3.24**). The promoter of SPRR3 did not have a single CpG island with length more than 50 CpGs.

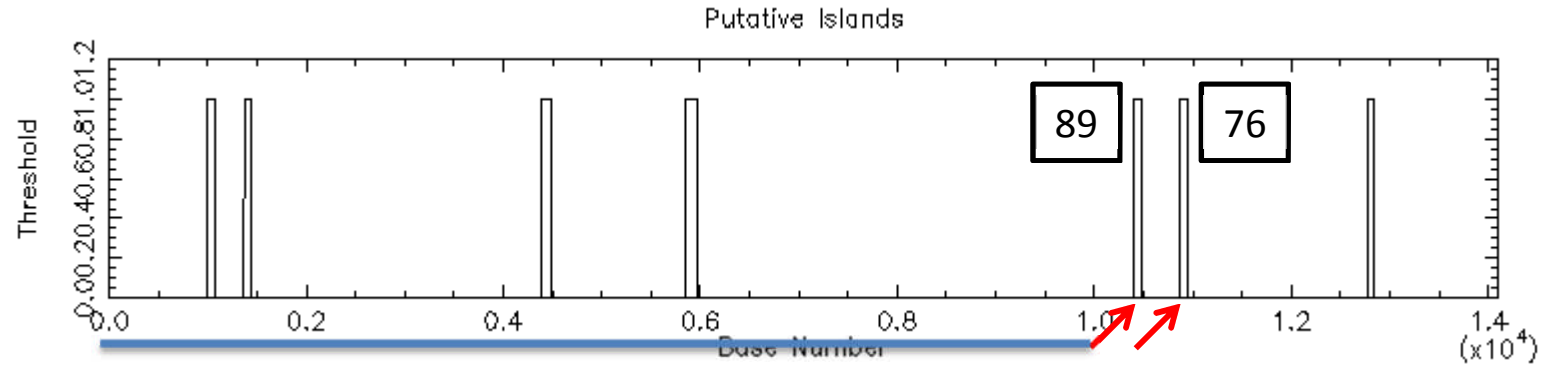


LCN2: 1kb promoter and a gene region

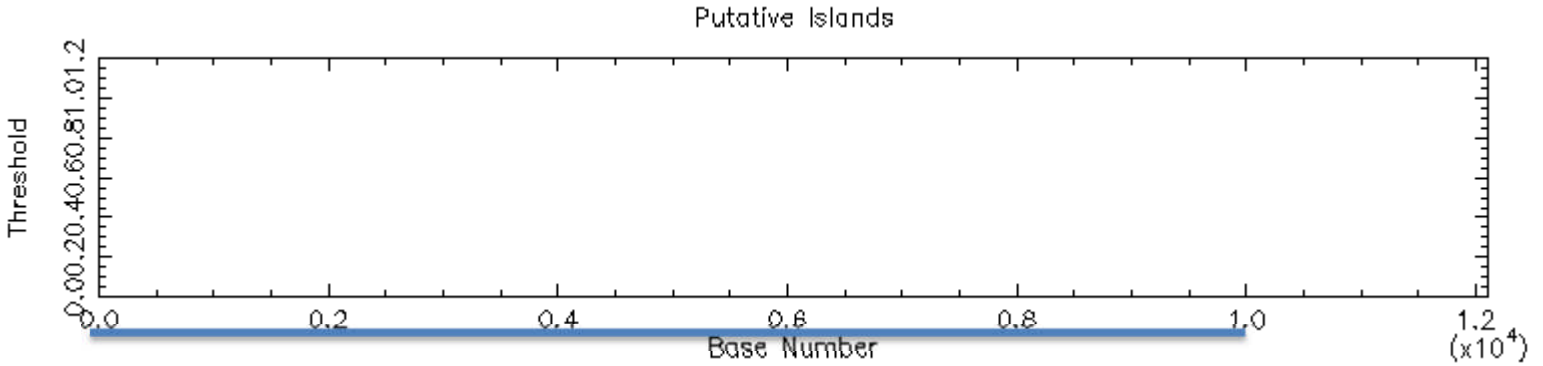


CEACAM6: 1kb promoter and a gene region

Figure I3.23: Identification of putative CpG islands at LCN2 and CEACAM6 genes. CpG islands were identified in a region 1kb prior to transcription start site (TSS) (blue line) and in a gene region using cpgplot. Red arrows indicate CpG islands closer to TSS. The number in the box indicates length of putative CpG island.



S100p: 1kb promoter and a gene region



SPRR3

Figure I3.24: Identification of putative CpG islands at S100p and SPRR3 genes. CpG islands were identified in a region 1kb prior to transcription start site (TSS) (blue line) and in a gene region using cpgplot. Red arrows indicate CpG islands closer to TSS. The number in the box indicates length of putative CpG island. There were no CpG islands detectable for SPRR3.

Thereafter, a panel of 8 prostate cell lines was treated with 5-aza-2′deoxycytidine (Azt) and trichostatin-A (TSA). Azt inhibits DNA methylation, and hence, promotes re-expression of genes repressed by hypermethylation. TSA inhibits histone-deacetylating enzymes, effectively promoting open chromatin. This change also promotes gene expression by allowing transcription factors to bind at gene promoters. If the genes are regulated with either DNA methylation or histone acetylation, remarkable changes (100-1000 fold) in gene expression are observed (Lin et al., 2001). We did not notice consistent and significant changes of that magnitude in gene expression after treatment with these agents for any of the genes in prostate cell lines (**Figure I3.25 and I3.26**). The pattern of gene up-regulation was relatively consistent and larger for S100p in Azt treated cell lines and in TSA treated cell lines for CEACAM6, suggesting that epigenetic regulation by DNA methylation and histone acetylation may be playing a partial role in their regulation. However, as we obtained much more promising results with studies involving the analysis of common regulation by transcription factors, and therefore, we did no further investigations related to the possibility of epigenetic regulation for the candidate genes' expression.

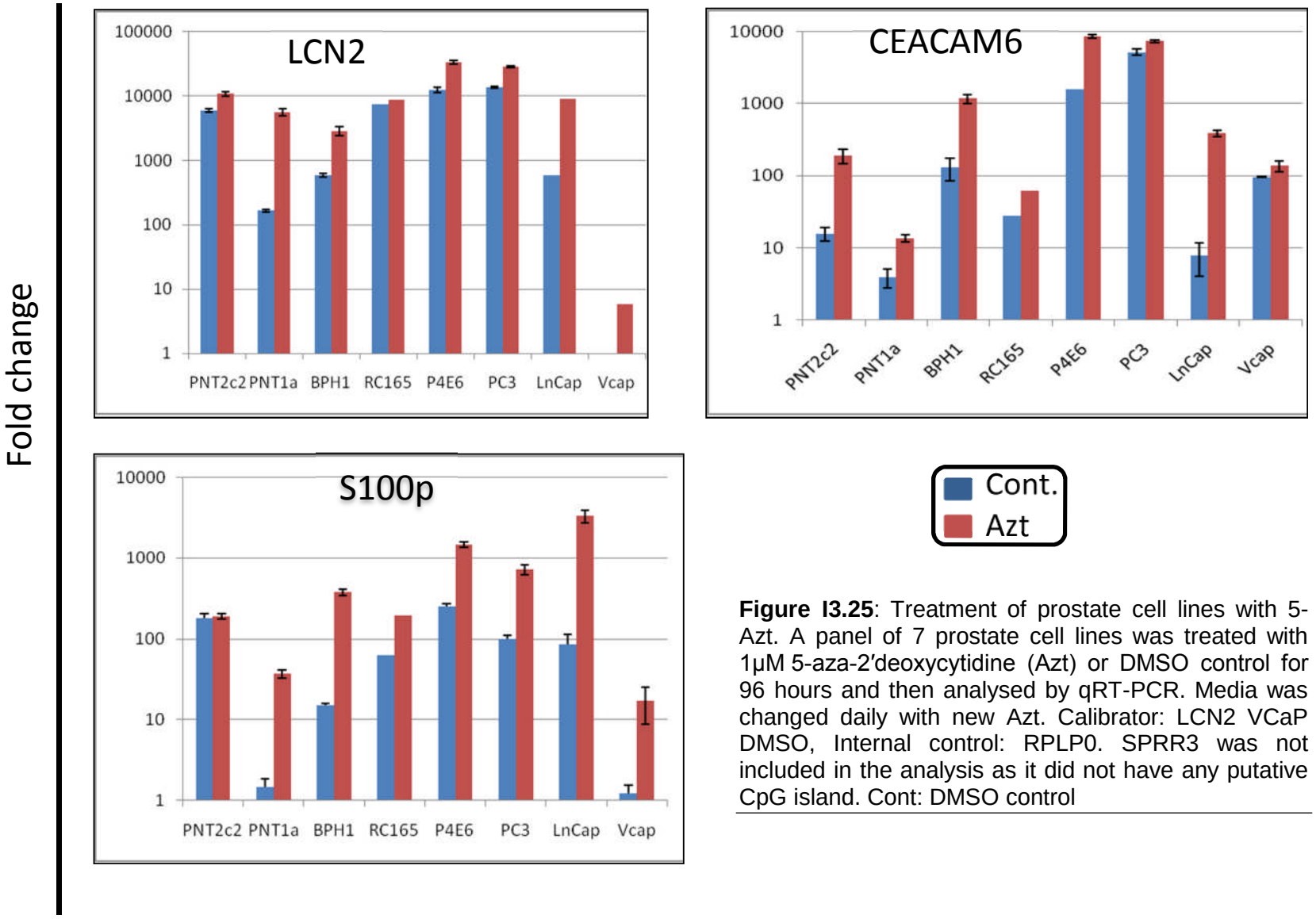


Figure I3.25: Treatment of prostate cell lines with 5-Azt. A panel of 7 prostate cell lines was treated with 1μM 5-aza-2′deoxycytidine (Azt) or DMSO control for 96 hours and then analysed by qRT-PCR. Media was changed daily with new Azt. Calibrator: LCN2 VCaP DMSO, Internal control: RPLP0. SPRR3 was not included in the analysis as it did not have any putative CpG island. Cont: DMSO control

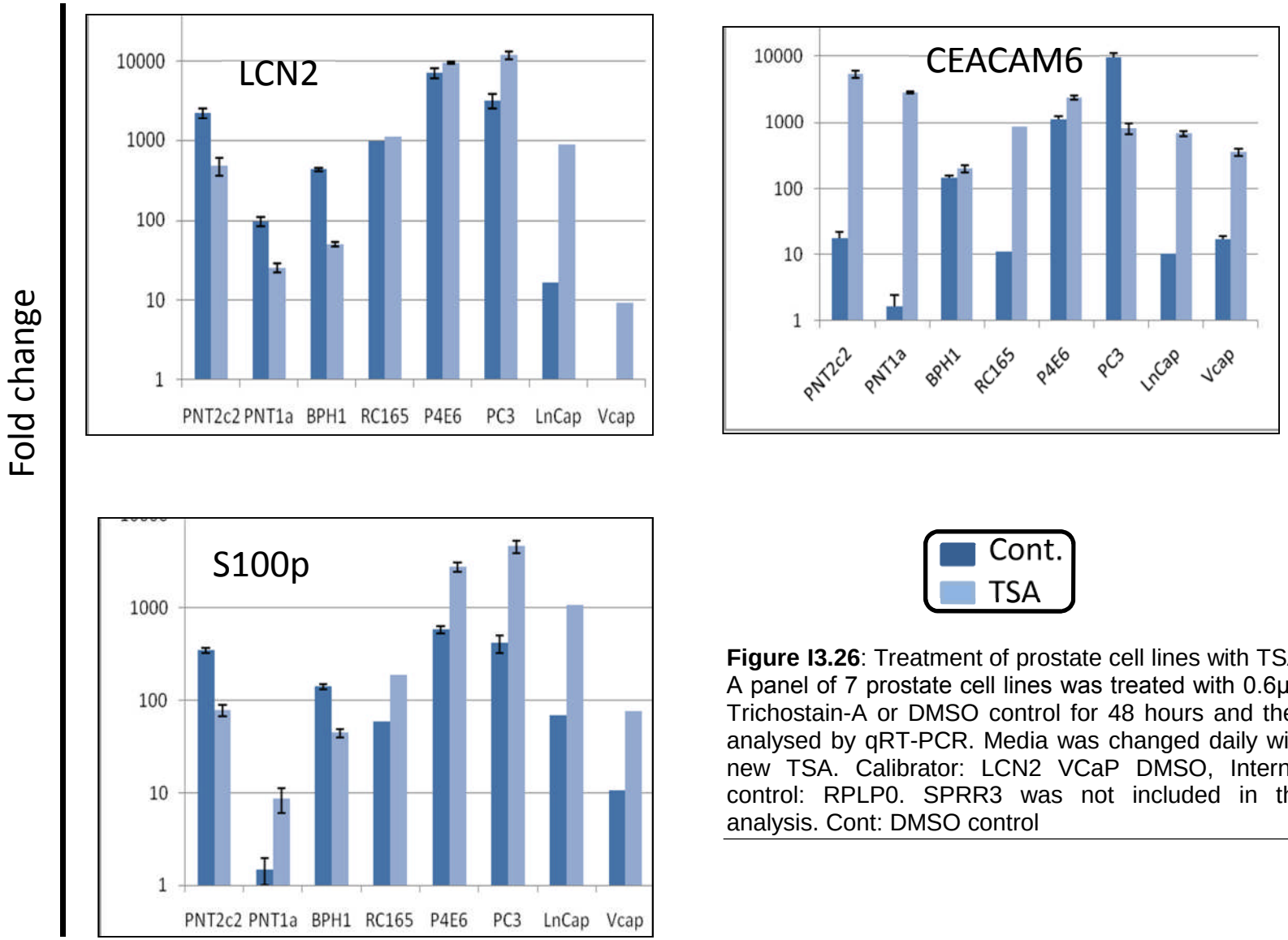
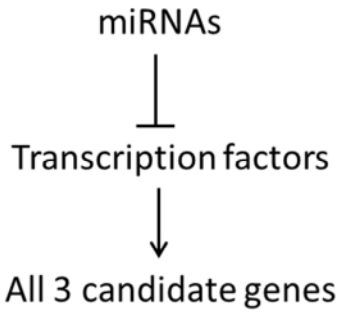


Figure I3.26: Treatment of prostate cell lines with TSA. A panel of 7 prostate cell lines was treated with 0.6µM Trichostain-A or DMSO control for 48 hours and then analysed by qRT-PCR. Media was changed daily with new TSA. Calibrator: LCN2 VCaP DMSO, Internal control: RPLP0. SPRR3 was not included in the analysis. Cont: DMSO control

I3.8 MicroRNA mediated regulation of the candidate genes

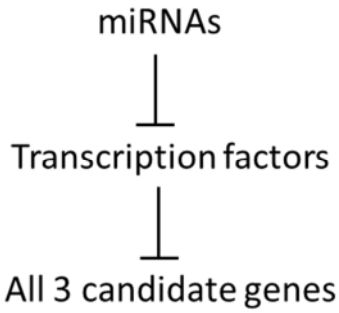
MicroRNAs are often suggested to negatively regulate a '*process'*, such as differentiation, by targeting multiple transcription factors (TFs) (Ambros, 2004). As the initial analysis suggested that LCN2, CEACAM6, and S100p could be co-regulated, we hypothesized that these three genes may be regulated by a single miRNA. However, there was no miRNA that could bind to the 3'UTRs of these 3 genes [according to analysis performed using ElMMo miRNA target prediction server (http://www.mirz.unibas.ch/ElMMo3/). Therefore, we attempted to identify miRNAs that can target majority of TFs that were predicted to bind on the promoters of all the candidate genes. Based on literature analysis and protein expression patterns (using the human protein atlas as a guide) (Uhlen et al., 2010), these common TFs fell into two categories: Group1 (**Figure I3.27**): those that potentially influence candidate gene expression positively (RXR, GR, STAT3, TAZ, VDR, SRF, PAX3, DMP1, and HSF1) and Group 2 (**Figure I3.28**): that potentially influence the candidate gene expression negatively (KLF4, KLF15, ZNF239, PAX6, MAZ, MZF-1, KCNIP3, and RREB1). Using ElMMo miRNA target prediction server (http://www.mirz.unibas.ch/ElMMo3/), it was identified that miR-548c can target all of the TFs from Group 1, whereas miR-188 can target RXR, VDR, GR, TAZ, and SRF (Gaidatzis et al., 2007). Our miRNA microarray analysis has indeed shown that these two miRNAs are repressed when the candidate genes are over-expressed. miR-128 can target the majority of TFs from the Group 2. This miRNA is over-expressed when the candidate genes are also over-expressed.



	Genes	TF	miRNA
SC	--	--	++
CB	++	++	--

Expected expression pattern

Figure I3.27: Expression pattern of miRNAs falling in Group 1. These miRNAs can inhibit the expression of transcription factors, which can positively regulate the expression of all the candidate genes. miR-548c and miR-188 fall in this category.



	Genes	TF	miRNA
SC	--	++	--
CB	++	--	++

Expected expression pattern

Figure I3.28: Expression pattern of miRNAs falling in Group 2. These miRNAs can inhibit the expression of transcription factors, which can negatively regulate the expression of all the candidate genes. miR-128 falls in this category.

In order to assess the effect of miR-188 and miR-548c on the expression of the candidate genes, committed basal cells enriched from 2 PPECs were transfected with respective miRNA mimics. The viable cells increased by about 60% 3 days after transfection of miR-188, whereas there was no significant effect on cells transfected with miR-548c (**Figure I3.29**). On the contrary, only miR-548c inhibited the expression of all the candidate genes significantly (45-90%) (**Figure I3.30**). Gene expression in scrambled siRNA control transfected samples was 3-5 fold lower than mock control sample, suggesting that the transfection protocol could be toxic to cells or transfection may have non-specific effects or there could be experimental errors. This experiment needs repetition to confirm the results and determine the cause of such an effect of mock/siRNA transfection.

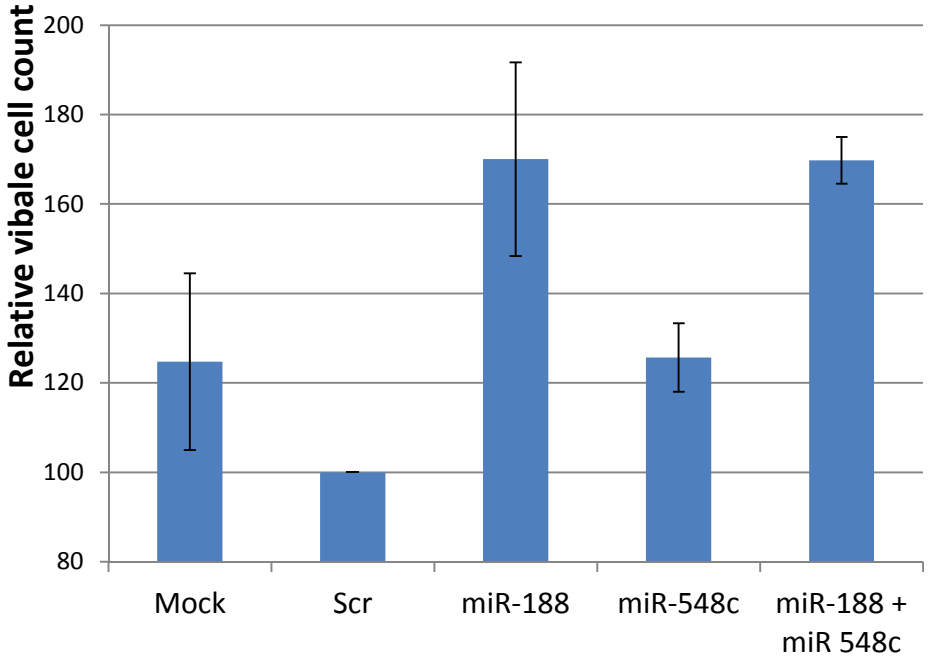


Figure I3.29: Transfection of BPH-derived PPECs with miR-188 and miR-548c mimics. 2 BPH-derived PPECs were transfected with 50nM of either miR-188 or miR-548c or with 30nM miR-188 and 30nM of miR-548c. Viable cell count was measured by trypan blue exclusion test after 3 days of transfection. Mock: mock transfected control, Scr: scrambled siRNA transfected control, N=1.

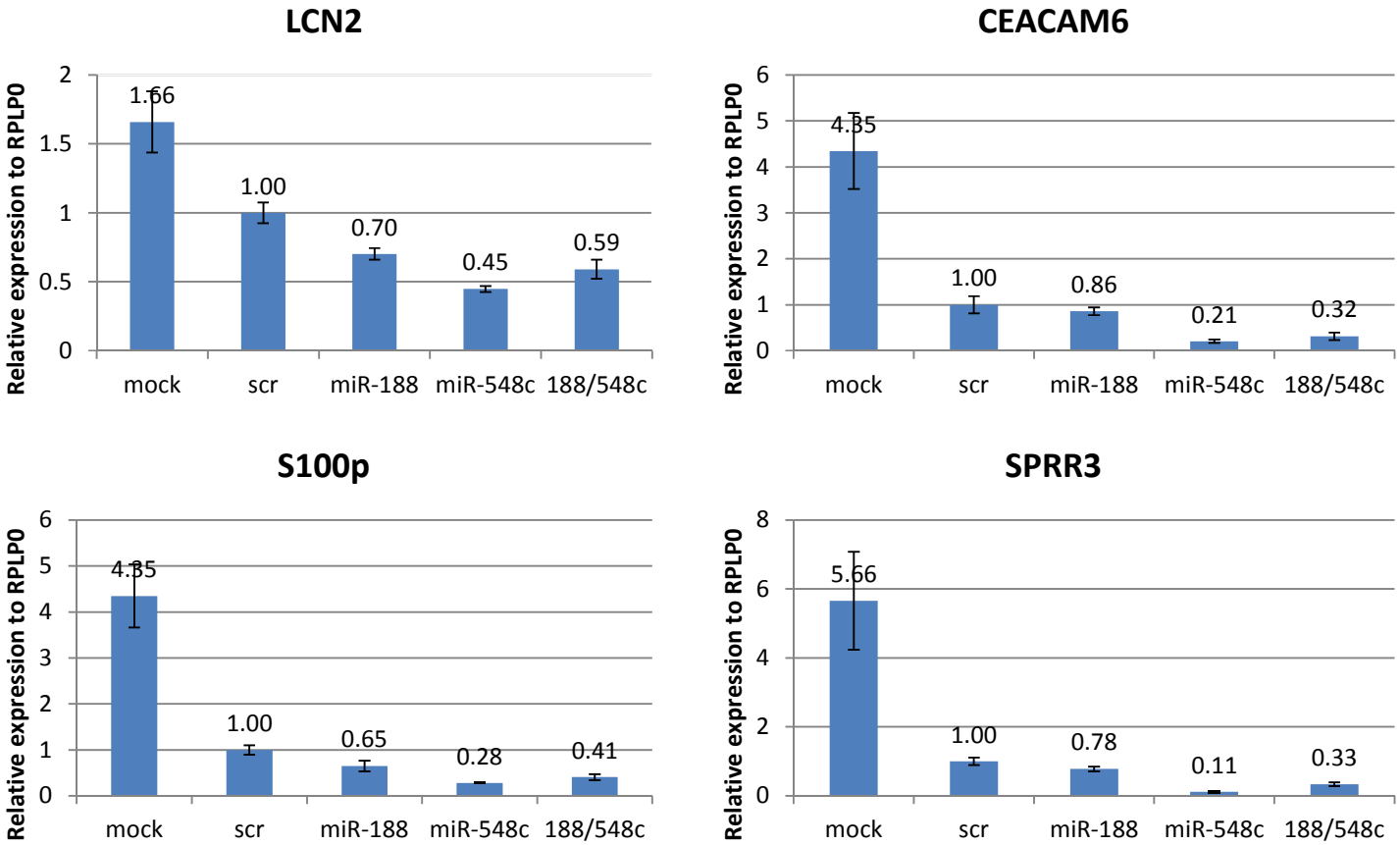


Figure I3.30: Transfection of BPH-derived PPECs with miR-188 and miR-548c mimics. A BPH-derived PPEC was transfected with 50nM of either miR-188 or miR-548c or with 30nM miR-188 and 30nM of miR-548c. Candidate gene expression was measured by qRT-PCR after 3 days of transfection. Mock: mock transfected control, scr: scrambled siRNA transfected control. Internal control: RPLP0.

I3.9 siRNA mediated knock-down of candidate genes

Functional studies were necessary to conclusively demonstrate the role of candidate genes in prostate stem cell regulation. To assess whether knockdown of these genes in differentiated committed basal cells leads to a change in cell fate, we first assessed the feasibility of siRNA-mediated knockdown of these genes in PPECs. We were able to obtain a significant reduction (40-60%) in the expression of all the candidate genes by 24 hours; however, the repression was most marked after 72 hours of transfection for all the genes (**Figure I3.31** and **32**). Knock down of these genes was then confirmed at the protein level by a western blot analysis (**Figure I3.33**). Interestingly, there were no significant differences in the mock and scrambled siRNA transfected samples in this experiment, even when similar reagents were used as those for miRNA transfection. This implied that controls used in the miRNA transfection studies need further confirmation to eliminate the possibility of experimental errors.

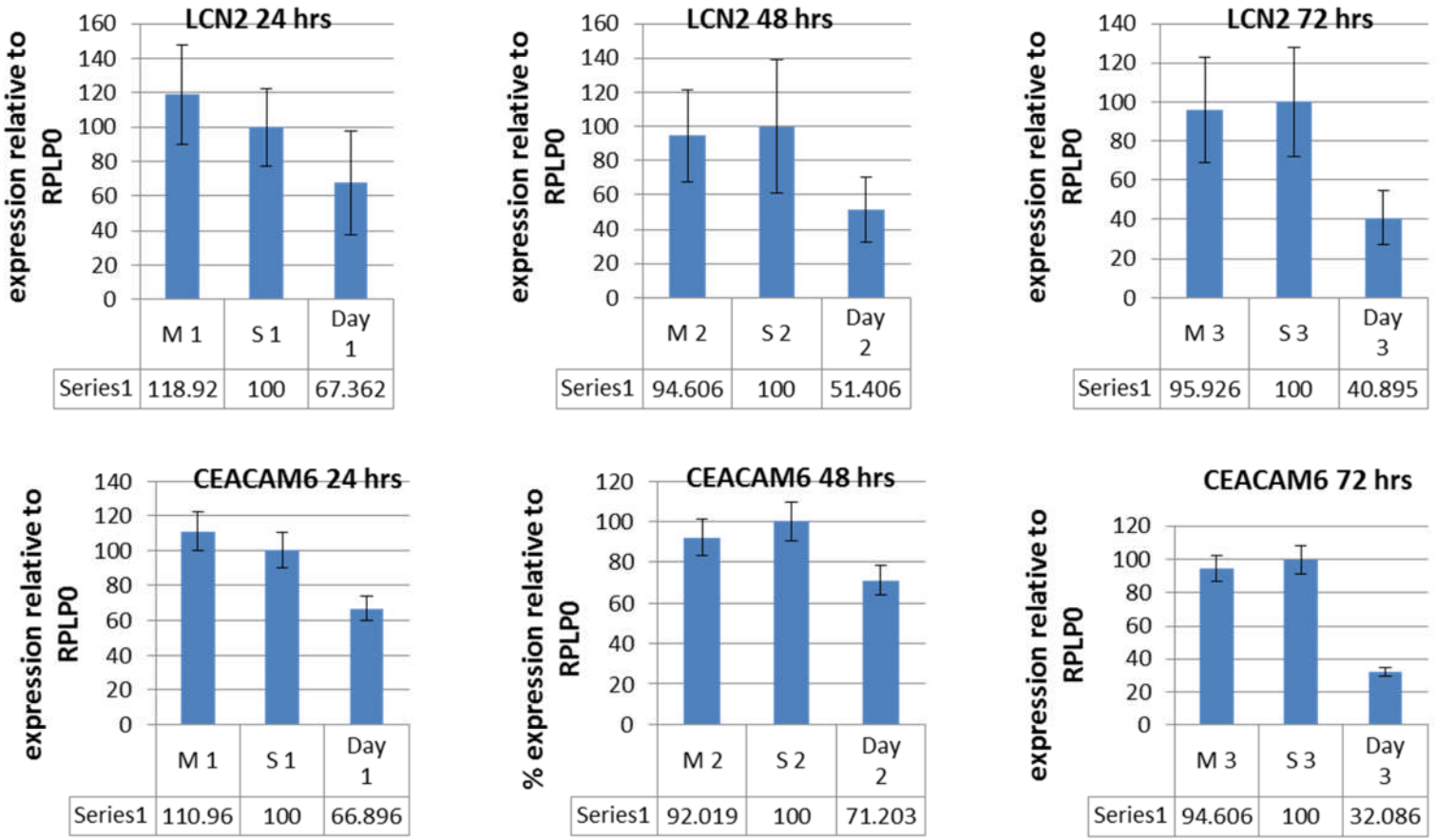


Figure I3.31: Transfection of BPH-derived PPEC with siRNAs for LCN2 and CEACAM6. A BPH-derived PPEC was transfected with 50nM of for LCN2 or CEACAM6 siRNA. The candidate genes' expression was measured by qRT-PCR after 24, 48 and 72 hours of transfection. Mock (M): mock transfected control, Scrambled (S): scrambled siRNA transfected control. Internal control: RPLP0

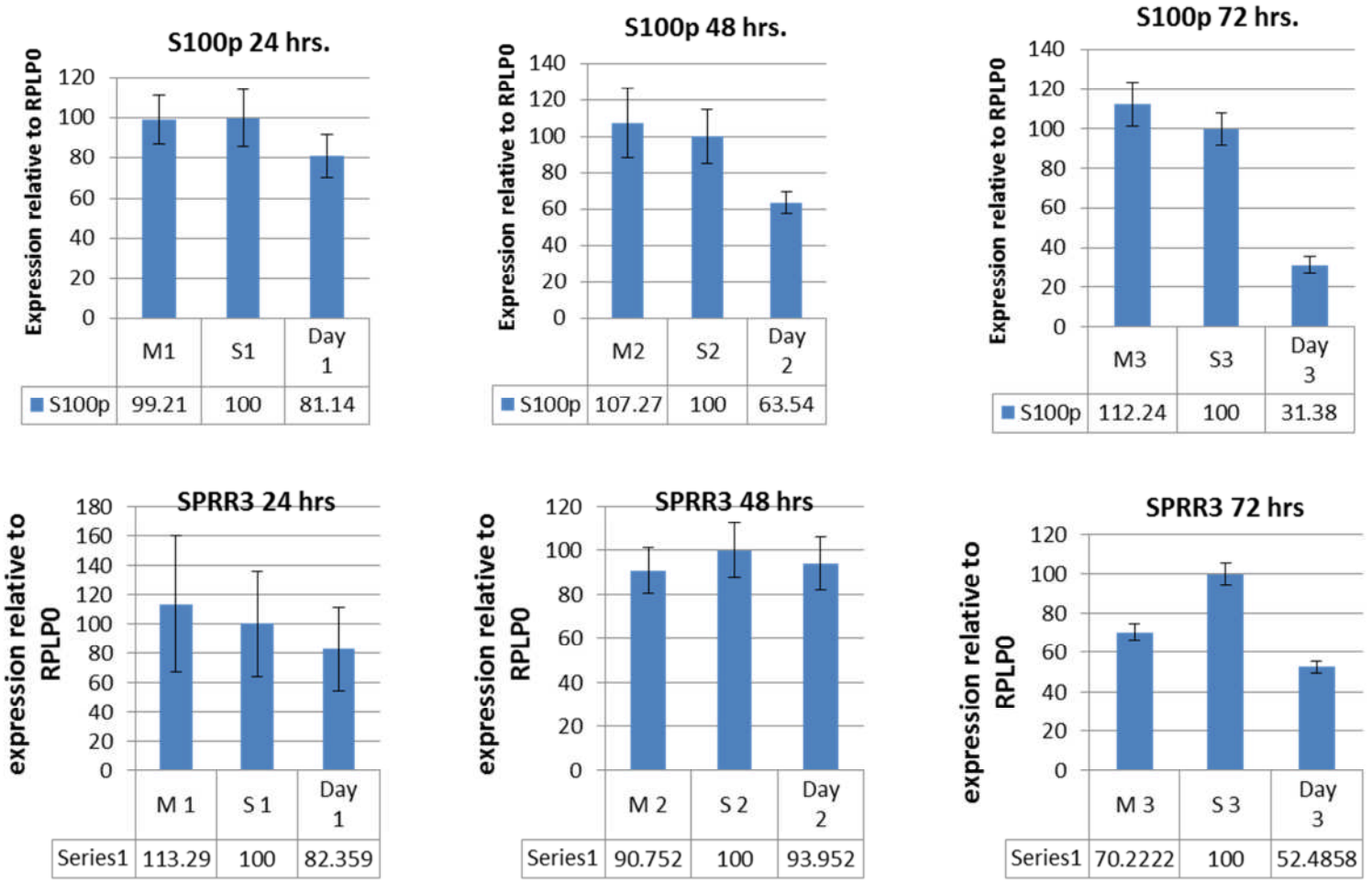


Figure I3.32: Transfection of BPH-derived PPEC with siRNAs for S100p and SPRR3. A BPH-derived PPEC was transfected with 50nM of for S100p or SPRR3 siRNA. The candidate genes' expression was measured by qRT-PCR after 24, 48 and 72 hours of transfection. Mock (M): mock transfected control, Scrambled (S): scrambled siRNA transfected control. Internal control: RPLP0

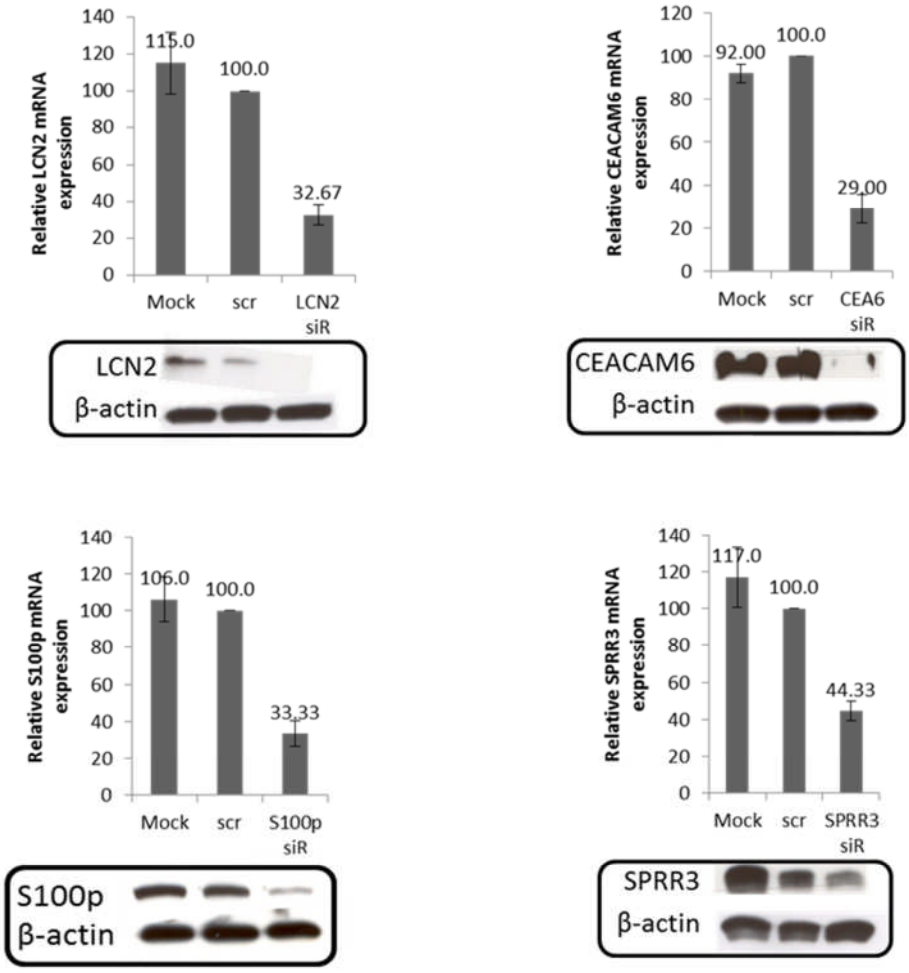


Figure I3.33: Transfection of BPH-derived PPECs with siRNAs for candidate genes. BPH-derived PPECs (n=3) were transfected with 50nM siRNAs for candidate genes and the candidate genes' expression was measured by qRT-PCR and western blot after 72 hours of transfection. Mock: mock transfected control, Scrambled: scrambled siRNA transfected control. Internal control: RPLP0.

Further assays investigating cell fate, such as more stem cell relevant changes in colony forming efficiency could now be employed to assess effects of knockdown of individual/all the candidate genes on differentiation.

SECTION I: 4. Discussion

In this investigation, we set out to identify genes that can regulate prostate stem cell differentiation. Selecting the candidate genes from the microarray data, we have discovered that LCN2, CEACAM6, and S100p may form a part of a co-regulated network. We therefore investigated the transcription factor mediated and epigenetic regulation of expression of these genes. The functional characterisation of LCN2, CEACAM6, and S100p can link transcriptional and miRNA-mediated prostate stem cell differentiation regulatory circuitry.

Identification of the candidate genes was based on a microarray experiment performed in our lab a few years ago (Birnie et al., 2008). The microarray process is a multi-step process. Each of these steps may suffer from technical and manual inadequacies, seriously damaging the quality of the raw microarray data. This could result in mis-reproduction of actual biological data during microarray statistical analysis (Cohen Freue et al., 2007). Therefore, it is essential to identify and eliminate arrays with low quality. For this purpose, we reanalysed previously published data from our lab, which compared gene expression profile of stem and differentiated committed basal cells enriched from benign and treatment naïve prostate cancer patients (Birnie et al., 2008). This data was reprocessed using the robust multi-array averaging (RMA) method as opposed to the MAS5.0 method used in the original paper, after confirming the good quality of all the microarray chips. It has been suggested that RMA is more consistent, specific and sensitive for differential gene expression analysis on microarray dataset comprising of multiple chips (Irizarry et al., 2003, Harr and Schlotterer, 2006) and therefore, it was preferred over MAS5.0. We used this reprocessed data and hypothesised that the differentiated cells over-express genes that are relevant for differentiation, to identify genes regulating prostate stem cell differentiation.

LCN2, CEACAM6, S100p, and SPRR3 genes were found to be among the top 35 over-expressed genes in differentiated cells and were chosen as candidate differentiation regulatory genes. All these genes are suppressed in stem and transit amplifying cells. The over-expression of these genes in committed basal cells and also in terminally differentiated luminal cells, suggests that these genes could be necessary for the initiation and the maintenance of differentiated phenotypes. The loss of this expression pattern in CRPC-derived primary prostate epithelial sub-populations and xenograft-derived sub-populations, where the differentiation process is aberrant (Gleave et al., 2005, Bonkhoff and Berges, 2010, Maitland et al., 2011), further indicate that these genes could be involved in the regulation of differentiation. The overexpression in luminal cells was also not preserved in prostate cell lines, suggesting that the expression in cell lines may not always match with primary sample derived sub-populations and the candidate genes could have diverse regulation and functions in cell lines. Nevertheless, the expression profile from primary prostate derived sub-populations strongly implicates LCN2, CEACAM6, S100p, and SPRR3 in prostate epithelial differentiation.

There is some evidence in other cell types and tissue models to indicate that these genes could be instrumental in the regulation of differentiation of normal and cancer stem cells. In mice, differentiating epithelial lens and kidney cells showed up-regulation of LCN2; whereas, SPRR3 was found to be up-regulated during epidermal differentiation during the process of skin repair (Hohl et al., 1995, Mishra et al., 2004, Medvedovic et al., 2006). LCN2 was also shown to be over-expressed precisely at the time of keratinocyte differentiation during mouse embryonic development (Mallbris et al., 2002). On the other hand, rat neuronal stem cells were suggested to have lower expression of S100p than their differentiated progeny (Sueoka and Droms, 1986) and indeed, lower expression of CEACAM6 has been proposed as a marker for human urothelial normal and cancer stem cells (He et al.,

2009). These findings, even though they did not explore detailed functional mechanisms, provided corroboration of the involvement of the candidate genes in the process of epithelial stem cell differentiation.

Similar to normal tissues, the overexpression of candidate genes was also correlated with the differentiation status of tumours. Literature reports showed that LCN2, CEACAM6, S100p, and SPRR3 are usually over-expressed in multiple human tumours (Arumugam et al., 2005, Han et al., 2007, Bolignano et al., 2010). But it is well established that in most early stage cancers, there is over-representation of the differentiated cell compartment. For example, the percentage of terminally differentiated cells in normal prostate is about 50%, whereas they constitute more than 99% mass of treatment naïve prostate tumour (Nagle et al., 1987, El-Alfy et al., 2000, Humphrey, 2007). The over-expression of candidate genes in such cases could merely be the consequence of an excessive content of differentiated cells. A more precise search of literature indeed revealed that only well differentiated tumours showed a higher expression of these genes, whereas matched poorly differentiated and aggressive tumours exhibited significantly lower expression (Scholzel et al., 2000, Moniaux et al., 2008, Cho and Kim, 2009). Additionally, LCN2 was found to be down-regulated in rat glioblastoma cells, where it was linked to the acquisition of chemoresistance (Zheng et al., 2009), suggesting the transformation of differentiated glioblastoma cells into more stem-like treatment resistant cells. Rapidly proliferating T47D breast cancer cells were also shown to acquire quiescence on S100p down-regulation (Ishii et al., 2005). Therefore, it is possible that LCN2, CEACAM6, S100p, and SPRR3 could be important for the maintenance of epithelial hierarchy in a wide variety of tissues in physiological and pathological scenarios.

LCN2, CEACAM6, S100p, and SPRR3 belong to diverse families of proteins, which mediate several cellular processes, ranging from immune regulation to cell differentiation. However, none of these genes are evolutionary conserved nor do their mouse knockout models (available only for LCN2) (Berger et al., 2006) show significant or obvious developmental defects. These observations suggest that these genes could individually be redundant, but together, may perform vital functions as a part of network regulated by key transcription factor/s. Literature analysis also pointed in this direction. For example, a retinoic acid agonist was shown to up-regulate LCN2, CEACM6, and S100p expression in sebaceous epithelium (Nelson et al., 2008). S100p can promote NF-kappa B signalling in NIH3T3 cells (Arumugam et al., 2004), whereas NF-kappa B-mediated signalling pathway positively influenced LCN2 expression in prostate cancer cell lines (Mahadevan et al., 2011). The overexpression of MMP9 was directly linked with overexpression of LCN2 (Coles et al., 1999, Nuntagowat et al., 2010), CEACAM6 (Duxbury et al., 2004), and S100p (Namba et al., 2009) in various cancer models. Such findings, together with our expression pattern analysis of these genes in published microarray experiments, indeed imply that LCN2, CEACAM6, and S100p could have similar functions and may also be co-regulated.

The analysis assessing transcription factor binding sites at the promoters of these genes further hinted towards the possibility of co-regulation. The promoters of all 4 genes contain androgen responsive elements (ARE), glucocorticoid responsive elements (GRE), and TEA domain family member 1 (TEAD1), which have well documented roles in differentiation and carcinogenesis, including the prostate (Long et al., 2005, McDevitt et al., 2007, Knight et al., 2008). VDR-RXR also has a binding site on the promoters of all of the candidate genes. It has been shown to be instrumental in the differentiation multiple epithelial and mesenchymal tissues (Botling et al., 1996, Bikle et al., 2003). The genes, which were suggested be co-

regulated in the compendium microarray analysis, LCN2, CEACAM6, and S100p, had binding sites for 22 common transcription factors at their promoters with consensus sequence homology > 80% and P < 0.001. Interestingly, the majority of transcription factors identified in this analysis were pro-differentiation transcription factors, such as androgen receptor (AR) (Long et al., 2005), vitamin D receptor-retinoic acid X receptor heterodimer (VDR-RXR) (Gudas and Wagner, 2011), GR (McDevitt et al., 2007), INSM1 (Lan and Breslin, 2009), BCL6 (Diehl et al., 2008), CEBPβ (Sankpal et al., 2006), MEF (Chen et al., 2000), and PAX6 (St-Onge et al., 1997). Analysis had also identified other transcription factors, which can regulate stem cell maintenance [NANOG (Cavaleri and Scholer, 2003) and GKLF/KLF4 (Kim et al., 2012b)], cell proliferation in normal and cancer tissue [SOX9 (Thomsen et al., 2008a), NF-κB (Rajasekhar et al., 2011), STAT1/3 (Lou et al., 2000, Kovacic et al., 2006), BACH2 (Green et al., 2009), and TEAD (Liu-Chittenden et al., 2012)], cancer cell metastasis [RREB1 (Melani et al., 2008)], chromatin status [SMARCA3 (Debauve et al., 2006) and RFX1 (Zhao et al., 2010)], and immune response [NFAT/NFAT5 (Pan et al., 2012)]. There is at least one paper for all these transcription factors that also implicates them directly in the regulation of stem cell differentiation. These findings indicate that, in addition to other functions, the candidate genes could be instrumental causal and/or effector genes in the regulation of the prostate epithelial differentiation.

Among all these transcription factors, retinoic acid (RA) receptors are particularly of greater interest, because: (i) there is a clear evidence for RA receptor binding sites on the promoters of all the genes, (ii) RA agonists can stimulate LCN2, CEACAM6, and S100p (Nelson et al., 2008), (iii) RA mediated signalling is instrumental in epithelial differentiation (Gudas and Wagner, 2011) and (iv) RXRγ null mice develops prostate epithelial metaplasia (Lohnes et al., 1995). Treatment with all-trans retinoic acid (ATRA) can also independently induce the expression of either of

LCN2 (Garay-Rojas et al., 1996, Cheepala et al., 2009), CEACAM6 (Ozeki and Shively, 2008), or S100p (Shyu et al., 2003) in other epithelial models. RA agonists can also promote prostate epithelial differentiation, as demonstrated by an increase in the luminal marker NKX3.1 in LNCaP cells (Jiang et al., 2006) and P21 in TRAMP mouse prostate (Huss et al., 2004). Furthermore, studies in rat prostate implied that RA and vitamin D3-mediated prostate epithelial differentiation could be mediated through autocrine production of TGF-β (Danielpour, 1996). Our results provide further evidence for the contribution of RA signalling, with or without Vitamin D3-mediated signalling, in the regulation of patient-derived prostate epithelial stem cell differentiation, via the candidate genes. However there are three important issues, which should be considered in relation to RA-mediated candidate gene regulation.

First, the effect of ATRA or a 9-cis-RA+1,25-dihydroxyvitamin D_3 combination on the expression of LCN2, CEACAM6, and S100p was not immediate (within 24 hours), suggesting that these agents may influence their expression through some intermediate. RA can, in fact, interact with other transcription factors, such as STAT1, which also have binding sites on the promoters of LCN2, CEACAM6, and S100p. There is clear direct evidence for ATRA mediated STAT1 transcriptional regulation (Gianni et al., 1997, Chelbi-Alix and Pelicano, 1999), and also for STAT1-RXRα functional complex formation (Trusca et al., 2012). The need for recruitment of such intermediates would delay the ultimate response. A second reason could be that the compounds used may not be sufficiently potent to elicit an immediate response. For example, it was shown that retinol could bind more strongly to the mouse LCN2 promoter than its hydrophilic counterpart retinoic acid (Chu et al., 1998). Additionally, the vitamin D_3 analogue EB1089 inhibits LNCaP cell growth more effectively than 1,25-dihydroxyvitamin D_3 and also formed a more synergistic combination with 9-cis-RA (Blutt et al., 1997). These possibilities remain to be explored. And the final issue is the remarkable SPRR3 downregulation after ATRA

or 9-cis-RA+1,25-dihydroxyvitamin-D_3 combination treatment. This observation clearly indicates that SPRR3 belongs to a different regulatory circuit than the other 3 candidate genes, and may have different regulation. Therefore, the focus on SPRR3 was reduced in further investigations assessing epigenetic regulation.

Another proposition to explain the common regulation for all of these genes was that they could be regulated by common epigenetic mechanisms. It is often noted that epigenetic alterations, such as DNA methylation and histone acetylation, play a major role in lineage specification (Hemberger et al., 2009). Previous studies have already established that S100p can be regulated by DNA methylation in prostate (Wang et al., 2007) and LCN2 in urothelial cell lines (Dokun et al., 2008). Treatment with DNA methyltransferase inhibitor (Azt) did not produce significant change in the expression of any of the candidate genes in a panel of prostate cell lines, with exception of S100p. Similarly; inhibition of histone acetylation could alter only CEACAM6 expression. As these investigations failed to provide a promising basis for co-regulation of LCN2, CEACAM6, and S100p, this line of investigation was discontinued.

Next, we attempted to investigate whether miRNAs could co-regulate candidate genes. The prediction algorithms however could not detect any common miRNA which could regulate LCN2, CEACAM6, and S100p expression. We then discovered that relatively unexplored (in prostate) miRNAs, miR-128, miR-188, and miR-548c could regulate most of the transcription factors, which we previously proposed for the regulation of the expression of candidate genes. Pilot experiments with miR-188 and miR-548c suggested that these miRNAs can indeed inhibit the expression of LCN2, CEACAM6, and S100p and transfection of these miRNAs into BPH PPECs could increase their proliferation. It is possible that after miR-188 transfection, the expression of LCN2, CEACAM6, and S100p is suppressed, pushing them towards the proliferating transit-amplifying phenotype from committed basal phenotype.

Detailed investigation of the role of miR-188 and miR-548c in prostate epithelial differentiation is therefore necessary to identify functional roles of these miRNAs. Nevertheless, these initial results implied that miRNA mediated regulation could play an important role in the regulation of the candidate genes.

Our investigations so far implied the association between the candidate genes and prostate stem cell differentiation. The conclusive functional evidence could only be obtained by assessing the effects of loss- or gain-of-function of these genes on prostate epithelial differentiation. Here, we provide evidence that these genes can be successfully knocked down by siRNA transfection in primary prostate epithelium. The absence of any obvious effect on cell morphology and cell growth kinetics suggested that knockdown of individual candidate genes may not be sufficient or efficient enough to alter cell fate. However, any specific impact in differentiation status was not tested directly, beyond on observations of morphology.

In summary, this project has identified a potentially interesting retinoic acid regulated gene-network of LCN2, CEACAM6, and S100p along with 3 miRNAs (miR-128, miR-188, and miR-548c), which probably can control prostate epithelial stem cell differentiation (**Figure I4.1**). Interference in regulatory pathway of these genes and miRNAs may be potentially exploited for prostate cancer differentiation therapy.

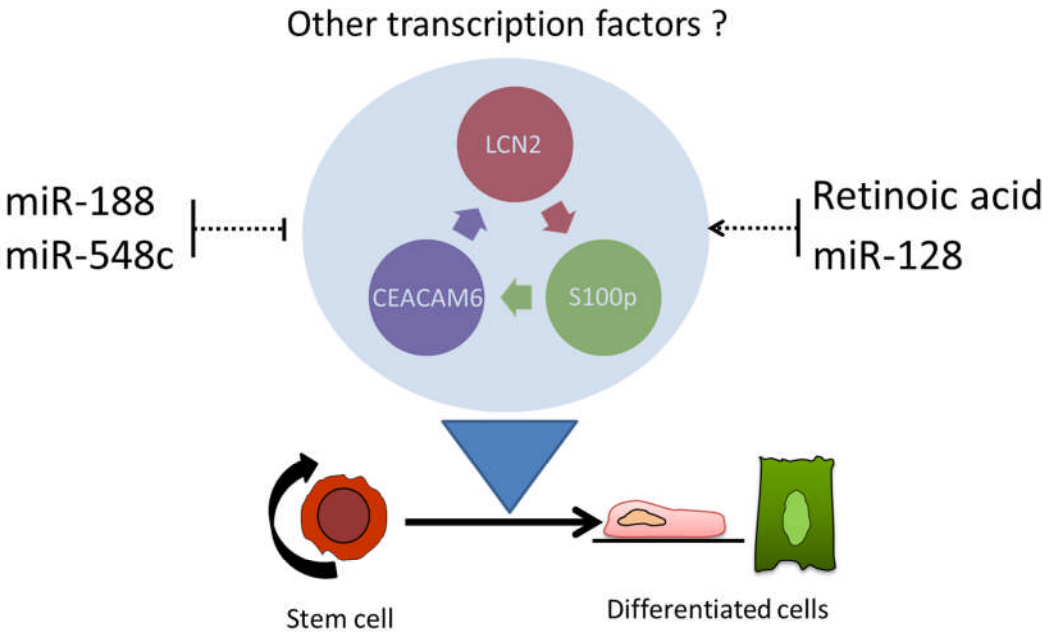


Figure I4.1: Hypothesis depicting one of the potentially important pathways in prostate stem cell differentiation.

Future work:

The regulation by RA receptors can be conclusively shown by chromatin immunoprecipitation. The co-regulation can also be further assessed by co-immunoprecipitation and chromosome conformation capture. The direct effect on prostate epithelial cell fate by modulating the expression of these genes or their regulators would conclusively establish the role of LCN2, CEACAM6, and S100p in the regulation of prostate epithelial differentiation.

Section II: The identification of the role of telomerase in BPH and prostate cancer

SECTION II: 1. Introduction

Since its discovery in 1984, telomerase has been an integral part of basic and translational research, with particular focus on aging, cancer, and stem cell behaviour (Greider and Blackburn, 1985, Blasco, 2005). Human telomerase is a ribonucleoprotein reverse transcriptase enzyme complex, which adds TTAGGG repeats to the 3'end of chromosomes (telomere regions) (Morin, 1989). Telomerase mediated telomere maintenance protects every chromosome against chromosomal fusion, recombination, and terminal DNA degradation (Blackburn, 2001). The majority of normal somatic cells do not express telomerase (Kim et al., 1994), and in these cells telomeres undergo cell division dependent erosion, subsequently leading to replicative senescence (Bodnar et al., 1998). This phenomenon of replicative senescence is one of the most important contributors in ageing. However cell types that have unlimited proliferative potential, such as germ cells and embryonic stem cells, circumvent the problem of replicative senescence by overexpressing telomerase (Flores et al., 2006). Often, precisely the same machinery is hijacked by cancer cells to acquire prolonged proliferative potential (Kim et al., 1994). Indeed, investigations exploring telomerase expression and functions in depth are becoming increasingly essential to understand these normal and pathological events.

II1.1 Telomerase enzyme complex: biogenesis and catalytic activation

Assembly of the active human telomerase ribonucleoprotein (henceforth, just telomerase) can be divided into three steps: (i) human telomeric RNA (hTR) and human telomerase reverse transcriptase (hTERT) synthesis and maturation, (ii) hTR-hTERT assembly leading to the catalytic activation, and (iii) recruitment to telomeres. The process of biogenesis is complex and can involve the interaction of

more than 30 proteins (Cohen et al., 2007). Only the major aspects of this process are described here.

The precursor of hTR is transcribed and undergoes internal modification after processing at its 3' end and capping on its 5' end to generate the mature and functional hTR (Feng et al., 1995, Collins, 2006). In addition to these modifications, the cellular accumulation of mature hTR also requires the binding of proteins such as dyskerin at 3'end (Mitchell et al., 1999, Pogacic et al., 2000, Meier, 2005). The 5' end folds into the tertiary pseudoknot (core) domain containing a template for telomere repeat addition and is also necessary for binding with the catalytic subunit of hTERT (Theimer et al., 2005). Until further structural conformations, the hTR accumulates in sub-nuclear Cajal bodies (Jady et al., 2004, Gallardo and Chartrand, 2008). The Cajal body localisation is instrumental role in the formation of hTR-hTERT complex and further recruitment of telomerase to telomeres (Cristofari et al., 2007).

The majority of tissues express hTR at various levels, but do not possess telomerase activity. Therefore, the availability of hTERT is considered as the rate-limiting step in telomerase activity (Meyerson et al., 1997, Weinrich et al., 1997). The lack of hTERT mRNA guarantees an absence of telomerase catalytic activity, but hTERT mRNA levels alone is not sufficient for the telomerase function. The hTERT mRNA is transcribed from the gene locus 5p15.33 and is subsequently spliced into at least 10 splice variants, some of which can have a dominant negative effect (Kilian et al., 1997, Colgin et al., 2000, Lincz et al., 2008). The active hTERT protein, unlike hTR, displays more diverse cell-cycle dependent sub-nuclear localisation, ranging from nucleolus to nucleoplasmic foci distinct from Cajal bodies (Yang et al., 2002, Tomlinson et al., 2006). Apart from the nucleus, 10-20% of the hTERT protein is also located in the mitochondria, proportion that can increase up to 80% under oxidative stress (Ahmed et al., 2008, Sharma et al., 2012).

Assembly of hTR-hTERT ribonucleoprotein and its recruitment to the telomeres is a very intricate process, which results in a compact hTR tertiary fold within the functional telomerase ribonucleoprotein complex. Moreover, post-translational hTERT modifications, such as phosphorylation, also influence telomerase catalytic activation (Liu et al., 2001b). The telomerase enzyme complex accumulates in the distinct nuclear bodies adjacent to Cajal bodies, which co-localise with telomeres in S phase of cell cycle (Jady et al., 2004, Tomlinson et al., 2006). Overall, at least 32 proteins can interact with telomerase enzyme components that may participate in its biogenesis, trafficking, activation, telomere recruitment and degradation (Cohen et al., 2007). However, the catalytically active human telomerase is composed of only two molecules each of hTERT, hTR, and dyskerin (Cohen et al., 2007). The activated telomerase enzyme is recruited to telomeres with the help of TCAB1 protein to add TTAGGG repeats to the telomeres by employing reverse transcription (Zhao et al., 2009) (**Figure II1.1**). The hTERT dependent reverse transcriptase activity is also present during mitochondrial DNA elongation. It was very recently shown that mitochondrial hTERT can function as hTR-independent reverse transcriptase; using mitochondrial tRNAs as template for reverse transcription (Sharma et al., 2012). These findings clearly suggest the multi-faceted role of telomerase in the overall development of an organism.

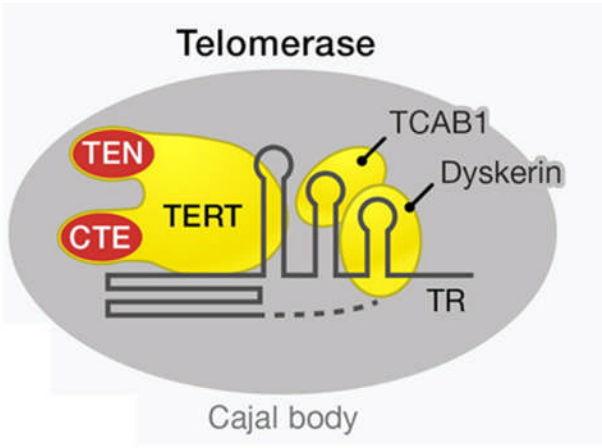


Figure II1.1: Schematic representation of telomerase ribonucleoprotein complex. The functional telomerase enzyme complex is composed of TERT, TR, and Dyskerin proteins and is located in sub-nuclear Cajal bodies. The TCAB1 protein facilitates the recruitment of telomerase to the telomeric ends of the chromosomes. Figure modified from (Noel and Wellinger, 2012).

II1.2 Functions of telomerase

Generation of telomerase null animal models and the consideration of human disorders due to telomerase insufficiency provided significant clues regarding functions of telomerase (**Table II1.1**). These studies showed that telomerase is not essential for survival of model organisms. However, homozygous telomerase mutations have not been observed in humans so far, suggesting that it may be essential for human survival. Nevertheless, investigations in all the organisms demonstrated defects in tissue homeostasis and cell proliferation. The interactions of telomerase with multiple signalling pathways highlight the tight regulation and widespread impact of telomerase mediated functions (**Table II1.2**). Here, the molecular basis for these observations is discussed.

Species	Homozygous null (-/-)	Heterozygous (-/+)	References
Budding yeast	Viable for up to many (>10) generations	No overt phenotype	Reviewed in (Lundblad, 2002)
Fission yeast	Delayed growth defect	No overt phenotype	(Haering et al., 2000)
Caenorhabditis elegans	Viable for up to 6 generations	No overt phenotype	(Cheung et al., 2006)
Arabidopsis	Viable for up to 10 generations	No overt phenotype	(Fitzgerald et al., 1999)
Mouse	Viable for up to 6 generations	No overt phenotype	(Liu et al., 2000)
Human	Not known (lethal?)	Various disorders including Dyskeratosis Congenita, aplastic anaemia and pulmonary fibrosis. Cancer predisposition	(Vulliamy et al., 2001, Fogarty et al., 2003, Yamaguchi et al., 2005, Armanios et al., 2007, Calado and Young, 2008)

Table II1.1: Summary of phenotypes generated by homozygous and heterozygous TERT deletion. Table taken from (Lansdorp, 2009).

Transcription factor	Role	Transcription factor	Role
AP-1	Repressor	TGF-β	Repressor
BRCA-1	Repressor	WT-1	Repressor
MAD1	Repressor	E2F-1	Repressor/Activator
MDM2	Repressor	Estrogen	Activator
Menin	Repressor	SP1	Activator
MZF-2	Repressor	STAT3	Activator
P53	Repressor	c-MYC	Activator
RAK/BRIT1	Repressor	U2F1/2	Activator
SIP-1	Repressor	Survivin	Activator
TAX	Repressor		

Table II1.2: Transcription factors which can regulate hTERT expression. Table modified from (Flores et al., 2006).

II1.2.1 Maintenance of telomeres

The primary function of telomerase is to maintain telomeric repeats at the end of human chromosomes. Telomeres are placed in position to avoid chromosomal abnormalities that can result from the end replication problem. The lagging strand is oriented in 5'-3' direction, so the replicating fork on the strand moves in the same

direction. However, the DNA replicating enzyme, DNA polymerase III, can function only in the opposite 3'-5' direction. So, replication on the lagging strand is initiated by multiple RNA primers, which are extended to Okazaki fragments and then joined together after removal of RNA primers (Sakabe and Okazaki, 1966, Ogawa and Okazaki, 1980). This method fails to replicate the gap left by the distal-most RNA primer, leaving daughter strand shorter (**Figure II1.2**) (Watson, 1972). In a context of regulation of cell-doubling potential, Olovnikov also independently proposed that daughter chromosomes lose terminal DNA sequences during replication (Olovnikov, 1973). He hypothesised that non-coding telogenes are located at the ends of the chromosomes. He also suggested that the exhaustion of these telogenes during repeated cell division underpin the eventual loss of cell division ability in culture, as suggested by Hayflick (Hayflick and Moorhead, 1961). Now, it is recognised that there are no telogenes, but rather evolutionary conserved guanine-rich TTAGGG DNA hexameric repeats (telomeres) exist to protect the ends of chromosomes and regulate cell division potential (Shampay et al., 1984, Moyzis et al., 1988, Allshire et al., 1989, Blackburn, 2000).

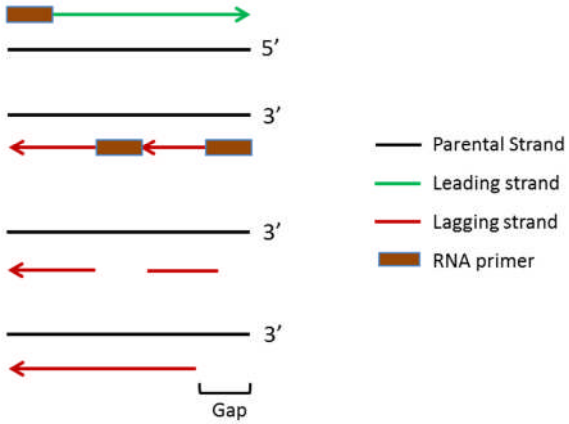


Figure II1.2: The end replication problem. During DNA replication, the lagging strand is initiated by multiple RNA primers. The extended products of RNA primers (Okazaki fragments) are then joined together to fill gaps. However the terminal primer gap can't be filled, leading to loss of terminal DNA sequences.

II1.2.1.1 The human telomeres

Human telomeres are nucleoprotein complexes of variable length. The length ranges from 5-15 kb, mainly based on age and organ (Moyzis et al., 1988, Allshire et al., 1989, Cross et al., 1989, Wright and Shay, 2005). Even in a single cell, telomere length varies significantly. The telomere length on chromosomal arms 17p, 19p, and 20q have been reported to be the shortest, whereas 5p, 3p, 4q, and 1p possess among the longest telomeres (Martens et al., 1998, Perner et al., 2003, Mayer et al., 2006, Samassekou et al., 2009). Furthermore, the length variations were also observed between homologous chromosomes, which can differ by up to 6kb in senescing cells (Surralles et al., 1999, Londono-Vallejo et al., 2001, Baird et al., 2003). The basis for these variable lengths is not understood so far. Nevertheless, at the end of each and every telomere, there is a single-stranded (ss) 3′ protrusion of ~12-300 bases, called the G-overhang (Wright et al., 1997, Zhao et al., 2008). These ss G-overhang curl back and are inserted into the double stranded (ds) telomeres to form a t-loop (**Figure II1.3**) (Griffith et al., 1999, Stansel et al., 2001). This arrangement protects the telomere end from being recognized as damaged DNA. The shelterin proteins are also instrumental in facilitating interaction of a plethora of other proteins with telomeres that can influence chromosomal end integrity (de Lange, 2005). Even though these proteins can protect telomeres from getting recognised as damaged DNA, 50-100bp of telomere is inevitably lost during each cell replication due to end replication problem (Harley et al., 1990). The average yearly telomere loss in adult human tissues is about 40-60 bp, which indicate extremely slow replication, or replication of very small subset of cells (Takubo et al., 2002).

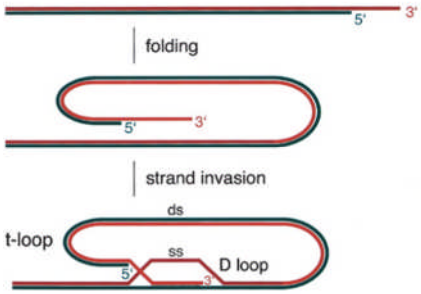


Figure II1.3: A schematic representation of the telomeric t-loop. When single stranded (ss) DNA of telomeric overhangs invade into double stranded (ds) telomeric DNA, t-loop is formed. It prevents telomeric DNA from recognised as damaged DNA. Modified from (de Lange, 2005)

II1.2.1.2 Regulation of telomere length

To control replication-associated telomere shortening, telomeres are subjected to a strict feedback regulation. The focus of this section is limited to what is known or hypothesized for human telomeres, as the positive and negative feedback loops that regulates telomere length can exhibit remarkable evolutionary flexibility (Palm and de Lange, 2008).

The t-loops (Wang et al., 2004), the RNA product of telomere transcription (Maicher et al., 2012), and sub-telomeric DNA methylation (Vera et al., 2008) has been proposed to variably regulate telomere length. But the central role in telomere regulation rests with the shelterin proteins (comprised of TRF1, TRF2, TIN2, RAP1, TPP1, and POT1) (de Lange, 2005). These proteins can only bind to the area of DNA where ss DNA and ds DNA lie in close proximity (Choi et al., 2011). This limitation gives them a clear specificity for telomeres. Proteins involved in DNA damage response, such ATM, Ku proteins and MRX complex interact with shelterin proteins to control the access of telomerase to telomeres (O'Connor et al., 2004, de Lange, 2005, Wu et al., 2007). The amount of shelterin proteins is directly proportional to the telomere length. So, on longer telomeres, these shelterin proteins can physically block the access of telomerase to the telomere end (**Figure**

II1.4) (de Lange, 2005). Once recruited, the telomerase enzyme complex contained in the Cajal body, associates itself with telomeres for about 10-40 minutes in the S-phase of cell cycle (Jady et al., 2004, Tomlinson et al., 2006). The ss G-overhang provides an anchor for telomerase RNA subunit to bind and extend telomeres. During this time, the enzyme successfully adds ~50-60bp in human cancer cell lines (**Figure II1.5**) (HeLa cells - cervical cancer cell line and H1299 cell – lung adenocarcinoma cell line) (Zhao et al., 2009, Zhao et al., 2011). This recent report demonstrating telomerase recruitment at about 70-100% of telomeres is in contrast with previous findings, which suggested that the recruitment occurs at only the shortest telomeres in the cells (Ouellette et al., 2000, Steinert et al., 2000, Bianchi and Shore, 2008). These investigations also showed that the length of shortest telomere, and not the average telomere length, determines the propensity for chromosomal instability and senescence.

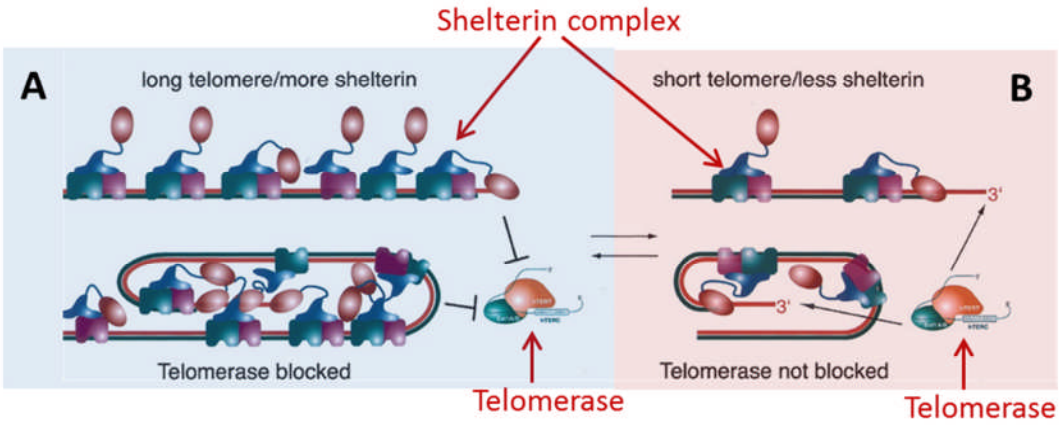


Figure II1.4: Shelterin proteins regulate telomerase recruitment to telomeres. Long telomeres have more shelterin proteins bound to it. These proteins prevent the entry of telomerase enzyme in the t-loop physically and through feedback-loop regulation (A). Once, telomeres become short, the amount of shelterin proteins bound to it also decreases allowing telomere-telomerase interaction (B). Modified from (de Lange, 2005).

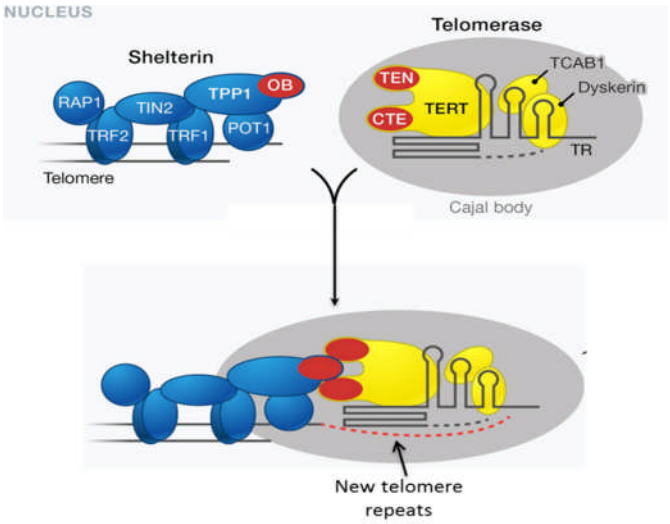


Figure II1.5: Telomere elongation by telomerase. Telomerase enzyme complex, which is accumulated in Cajal bodies, is recruited to telomeres with the help of TCAB1 protein. The TERT subunit binds with TPP1 protein of shelterin complex and telomeric RNA overhang provide anchorage for the TR component. Once telomerase-telomere come in proximity to each other, this association can last for about half-an-hour. Modified from (Noel and Wellinger, 2012).

II1.2.1.3 Consequences of telomere shortening

In the majority of adult somatic human cells, telomerase is absent. It is believed that when one or more telomeres in a cell are reduced to a critical length, cells undergo replicative senescence (Cong and Shay, 2008). In senescence, the damaged telomeres signal through ATM kinase mediated P53 activation (**Figure II1.6**). This process culminates in the up-regulation of the cell cycle inhibitors P21, p16 and Rb. The final outcome is an irreversible cell cycle arrest, which leads to senescence (Ouellette et al., 2011). This phenotype can also be induced by oxidative stress, certain oncogenes, and DNA damage signals. Senescent cells, while in arrest, remain metabolically active and even produce paracrine secretory factors to influence the surrounding tissue (Shay and Wright, 2005). The classical features of senescence are mentioned in (**Figure II1.7**). The lysosomal fraction of the cell, which contains β-galactosidase enzyme, increases significantly in senescent cells. Detection of this intense β-galactosidase staining under acidic conditions is the commonest marker for the detection of the senescence (Schmitt, 2007). Reduction

of telomere length to the critical level induces senescence, but the critical length determination depends on multiple factors. The variation in 'critical length' of telomeres that has to be maintained also varies upon the availability of sheltering proteins (Karlseder et al., 2002). In pathological cases, where cells continue to divide even in the presence of critically short telomeres, cells go through a crisis period. The outcome of this crisis depends upon successful re-expression of telomerase. If it is expressed, cells undergo immortalisation or oncogenic transformation, and if not, death by apoptosis.

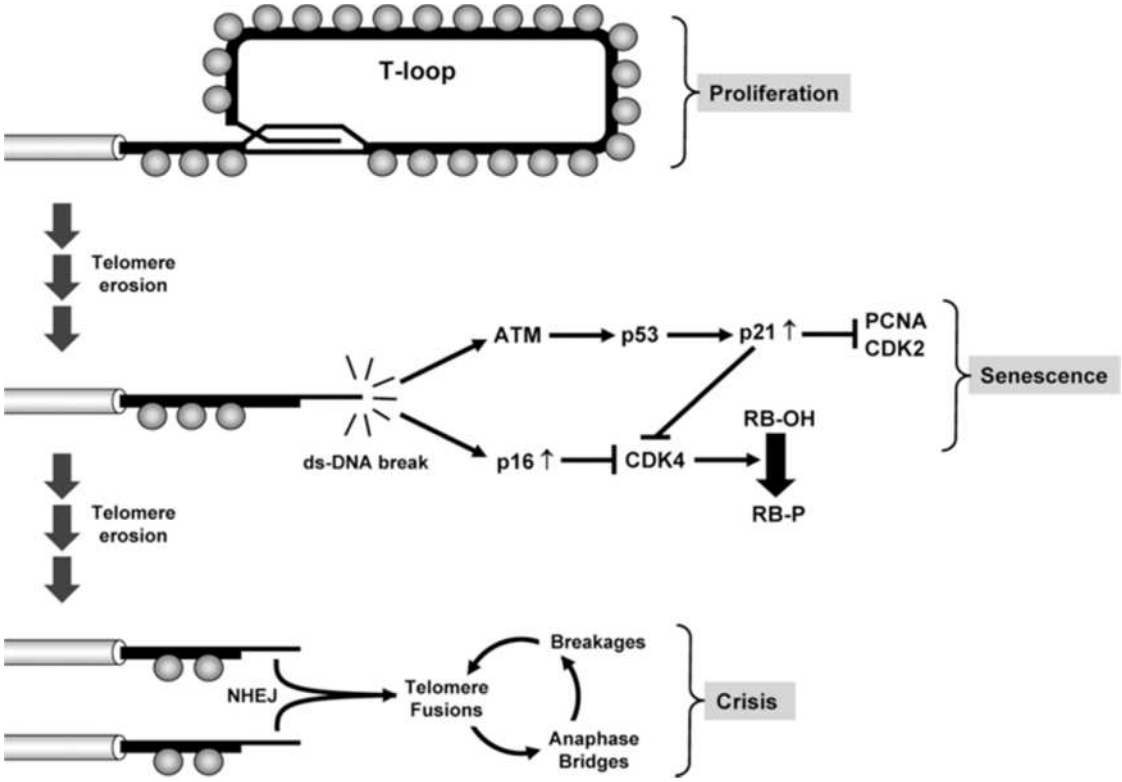


Figure II1.6. Induction of senescence and crisis as a function of telomere attrition. With every cell division, telomeres lose 50-100bps and shelterin proteins (grey) bound to them. This leads to unwinding of t-loop. The shorter and linear telomere is then recognised as a ds-DNA break. In the absence of any mutation in cell cycle checkpoints, this activates cell cycle inhibitors and the senescence pathway. When one of these mutations is present, the cell can ignore DNA damage signals. Telomeres serve as substrate for NHEJ break-bridge-break cycle and chromosomal fusion. This crisis is characterised by P53 independent apoptosis. Rarely, one of the clones in crisis acquires stability by expressing telomerase. Modified from (Ouellette et al., 2011).

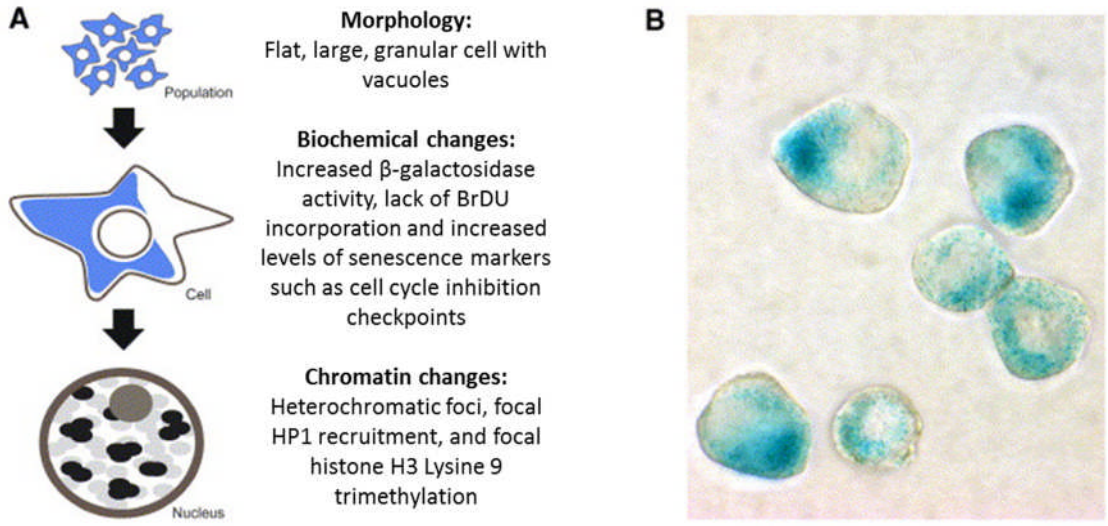


Figure II1.7: Characteristics of senescent cells. A: Schematic representation of senescent cells with classical features. B: senescent cells stained for β-galactosidase marker (blue). Modified from (Schmitt, 2007).

II.2.1.4 Relationship between telomere length, telomerase activity and proliferative potential of cells

Overall, it is quite clear that telomerase is essential for maintaining telomere length, which in turn is instrumental in the regulation of cell proliferation potential. However, multiple lines of evidence suggest that telomere length and telomerase activity do not always correlate. First, no direct correlation was found between telomerase activity, telomere length and the proliferative potential of human tissues (Hiyama et al., 1996, Wynn et al., 1998, Yui et al., 1998, Hodes et al., 2002, Masutomi et al., 2003, Yanada et al., 2006). Second, multiple investigations failed to find any correlation between telomere length and telomerase activity in newly established hES cell lines e.g. (Amit et al., 2000). Third, 10-15% of tumours with a very high proliferation index did not show any telomerase activity. And lastly, highly proliferative induced pluripotent stem (iPS) cells with long telomeres could be successfully generated from cells that lacked telomerase activity due to mutated hTERT/hTR (Agarwal et al., 2010, Batista et al., 2011). These observations led to the conclusion that the presence of detectable telomerase activity is neither

sufficient nor necessary for unlimited or immortal cell replication via telomere elongation. In cases where telomerase is barely detectable, telomerase may just *temporarily* extend proliferative potential or perform some extra-telomeric function. Cells can utilise the non-telomerase mechanisms, called alternative lengthening of telomeres (ALT), in the absence of telomerase.

II1.2.1.5 Alternative lengthening of telomeres (ALT)

The ALT has been observed in many in vitro cell culture models, but the precise mechanisms underlying it remain unknown (Cesare and Reddel, 2010, Shay et al., 2012). Recombination based telomere elongation (Dunham et al., 2000, Henson et al., 2009, Muntoni et al., 2009) and telomeric sister-chromatid exchange (Bailey et al., 2004) are two of the likely propositions for ALT (**Figure II1.8**). The telomeres of ALT cells retain most of the characteristic canonical telomeric attributes, but display one or more of the following features: heterogeneous telomere lengths (Bryan et al., 1995), increased numbers of DNA damage response foci at telomeres (Cesare et al., 2009), an abundance of extra-chromosomal telomeric DNA (ds t-circles, ss circles, and linear DNA) (Tokutake et al., 1998, Wang et al., 2004, Cesare et al., 2009), the presence of ALT-associated promyelocytic leukemia bodies containing telomeric DNA (Yeager et al., 1999), and a reduction in the binding saturation of shelterin proteins at telomeres (Cesare and Reddel, 2010). The simultaneous presence of ALT and telomerase has also been noted in human cells in culture (Cerone et al., 2001, Johnson et al., 2005), but the functional co-existence of these two mechanisms in vivo has not been identified so far. There are some hints that shelterin proteins (Celli et al., 2006, Wu et al., 2006) and methylation of sub-telomeric regions (Blasco, 2007) may inhibit ALT in the presence of telomerase. Interestingly, experiments with the ALT immortalised GM847 cell line showed that ectopic expression of mutated hTERT, which was unable to form a functional telomerase complex, was necessary for oncogenic transformation (Stewart et al.,

2002). This and similar other investigations indicated that telomerase (or hTERT) may have some significant extra-telomeric functions.

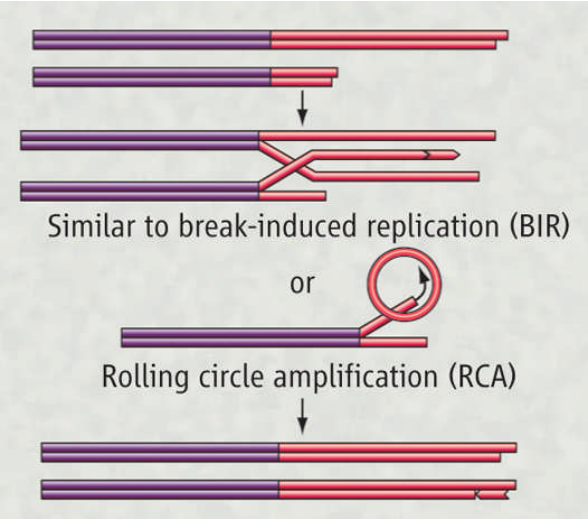


Figure II1.8: The mechansims for alternative lengthening of telomeres (ALT). In ALT a telomeric DNA (red) template is copied. The template may come from nonhomologous chromosome, or it could include extrachromosomal telomeric DNA. The extrachromosomal telomeric DNA can be in circular (illustrated) or linear form and can be derived from another region of the same telomere via loop formation, or the telomere of a sister chromatid. Modified from (Shay et al., 2012)

II1.2.2 Extra-telomeric functions of telomerase

Telomerase is not just an enzyme that can elongate telomeres, but its subunits can also perform a multitude of cellular functions. These functions are independent of its telomere maintaining activity and are primarily carried out by the TERT subunit. It is essential to consider extra-telomeric functions of telomerase in detail, to comprehend the role of telomerase in cancer and aging more clearly.

II.1.2.2.1 Telomerase and DNA damage response

Telomerase, especially the hTERT subunit, is intimately linked to the DNA damage response. Cells lacking hTERT exhibit higher radiosensitivity, reduced DNA repair ability, and fragmented chromosomes (Masutomi et al., 2005). Telomerase is physically associated with proteins that are instrumental in DNA damage response. The enzyme also interacts with the DNA replication protein primase, which is

associated with DNA repair (Ray et al., 2002). Telomerase can also add telomere hexamers to join broken DNA ends (Schulz and Zakian, 1994, Myung et al., 2001). These associations and observations indirectly suggest an important role for telomerase in DNA repair. Additional gain-of-function studies demonstrated a more direct relationship between telomerase and DNA repair. The ectopic overexpression of hTERT enhanced genomic stability and DNA repair in these studies (Sharma et al., 2003). Correspondingly, telomerase inhibition resulted in impairment of cellular response to DNA double strand breaks and nucleotide excision repair capacity (Masutomi et al., 2003, Shin et al., 2004). One of these investigations also showed that telomerase is expressed transiently in S-phase of cell cycle to protect karyotypic stability by capping chromosomes and resetting chromatin, without adding even a single telomere repeat (Masutomi et al., 2003). This can explain telomerase expression in adult human tissues, where cell division dependent telomere shortening is observed even in the presence of telomerase. The DNA repair function of telomerase is under the regulation of DNA damage response pathway itself, for example via Pif1, to prevent unwarranted healing of broken DNA ends (Makovets and Blackburn, 2009). Support for the role of telomerase in DNA repair is increasing day by day, but not all studies agree with this notion (Kampinga et al., 2004, Bates et al., 2005), suggesting that this aspect of telomerase function needs additional direct functional evidence.

II1.2.2.2 Telomerase and gene regulation

Multiple lines of evidence signify that telomerase regulates processes such as metabolism, cell cycle, and proliferation. Several independent transcriptional analyses with multiple human cell lines have revealed that hTERT over-expression or inhibition seriously affect gene expression patterns (Cong and Shay, 2008). Telomerase can influence gene expression patterns by: (i) direct influence on key genes (ii) chromatin manipulation (iii), interaction with signalling pathways, and (iv)

endogenous siRNA production. Gene set enrichment analysis revealed that TERT controls tissue progenitor cells via transcriptional regulation that converge on the c-MYC and WNT pathways (Choi et al., 2008). Indeed, hTERT can function as a cofactor in the β-catenin transcriptional complex to modulate WNT targets in human cancer cells (HeLa cells) (Park et al., 2009) and can also up-regulate c-MYC expression (Wang et al., 2000). Overexpression of the hTERT subunit also results in concurrent inhibition of P53, P21, and TGF-β signalling pathway, in addition to activation of the E2F/RB pathway and DNA methyl transferase 1 (Xiang et al., 2002, Young et al., 2003, Geserick et al., 2006). The hTERT mediated positive regulation of WNT and MYC pathways and inhibition of P53 strongly suggest that overexpression of telomerase favours cell survival and proliferation. Interestingly, some of these genes and epigenetic mechanisms can also regulate hTERT expression, resulting in auto-regulatory feedback loops. Recent investigation showed that WNT itself could regulate TERT expression in several different stem cell types (Hoffmeyer et al., 2012). Similarly, c-MYC (Wang et al., 1998), P53 (Cong et al., 2002), TGF-β signalling pathway (Cassar et al., 2010), and histone methyltransferase (SMYD3) (Liu et al., 2007a) all can regulate hTERT transcription. An additional mechanism by which telomerase could regulate gene expression is the synthesis of ds DNA, which can be converted into siRNAs by endogenous small interfering RNA (siRNA) processing machinery. The TERT subunit with the RNA component of mitochondrial RNA processing endoribonuclease (RMRP) can act as an RNA dependent RNA polymerase (RDRP) to produce ds DNA (Maida et al., 2009). The Dicer enzyme processes this ds DNA into siRNA, which controls RMRP levels. It is likely that the TERT–RMRP complex may amplify other small non-coding RNAs and thereby regulate wider gene expression. This RDRP activity can potentially also be vital for telomerase-mediated mitochondrial DNA repair and apoptosis resistance.

II1.2.2.3 Telomerase and apoptosis

It has been widely reported that telomerase inhibition can induce cell death through telomere attrition, but recent investigations have identified non-telomeric functions of telomerase that can influence apoptosis. Two clear lines of thought exist, proposing telomerase to be pro-apoptotic or anti-apoptotic (Cong and Shay, 2008). For the pro-apoptotic role, it was shown that oxidative stress induces nuclear export of TERT, probably to mitochondria (Haendeler et al., 2004). Telomerase in mitochondria can sensitise mitochondrial DNA to oxidative damage, leading to apoptosis (Santos et al., 2004). Mutations in the N-terminal peptide responsible for mitochondrial localisation of TERT resulted in the loss of mitochondrial oxidative damage, without affecting telomere elongation ability of telomerase (Santos et al., 2006). However, the molecular mechanisms for this pro-apoptotic action have not been identified so far. At the same time, other evidence supports the anti-apoptotic role of telomerase. For example, human fibroblasts overexpressing telomerase resist apoptosis but not senescence (Gorbunova et al., 2002, Lee et al., 2008b). This may be due to inhibition of the mitochondrial apoptotic pathway via Bax activation (Zhang et al., 2003a, Massard et al., 2006). More recent evidence augments the support for non-telomere dependent role of telomerase in the direct inhibition of apoptosis (Lee et al., 2008b). Apart from BAX activation, the other possible mechanism for the anti-apoptotic function could be telomerase-mediated synthesis of mtDNA that can minimise the impact of oxidative damage (Sharma et al., 2012). Due to this role, oxidative stress can induce nuclear export of TERT to mitochondria. In summary, more compelling evidence exists for anti-apoptotic function of telomerase, but two independent opposing pathways may also exist for telomerase-mediated apoptosis regulation.

In summary, maintenance of telomeres is the primary function of telomerase. Multiple transcription factors can also interact and modulate telomerase expression,

which in turn can influence several key signalling cascades. These interactions result in a widespread impacts on cell proliferative potential through telomere maintenance, DNA repair, apoptosis and stem cell maintenance. The higher expression of telomerase in hES cells (Thomson et al., 1998) and in 90% of the cancers (Shay and Wright, 2006) reinnforces the notion that telomeric and extra-telomeric function of telomerase converge to regulate stem cell behaviour and cell proliferation. The identity and location of telomerase expressing cells provide further evidence for the role of telomerase in the regulation of these processes.

II1.3 Telomerase expression and activity

In the early stages of telomerase research, a very sensitive telomeric repeat amplification protocol (TRAP) assay was developed to measure telomerase activity even from 1-10 cells (Wright et al., 1995). The TRAP assay involves the preparation of a protein extract by cell lysis and addition of a primer and dNTPs. If telomerase is present in the protein extract, it uses the primer as an artificial chromosome and adds telomeric repeats to it. The reaction product is then amplified by PCR and detected by qPCR, ELISA or gel based techniques (Fajkus, 2006). Some novel modifications and other approaches such as in-cell TRAP assay (Ohyashiki et al., 1998), in-situ hybridisation analysis for hTR (Paradis et al., 1999), and immunohistochemical staining for hTERT (Frost et al., 2000), in addition to qRT-PCR based detection of hTR/hTERT mRNA, were also developed to determine telomerase activity or expression. These techniques enabled the quantitative and semi-quantitative determination of telomerase activity/expression in wide variety of tissue samples.

II1.3.1 Telomerase expression/activity in development

During mammalian development, both telomerase activity and telomere length vary drastically on either side of the fertilisation process. The mature human gametes have short telomeres and no telomerase activity, but both of them increase dramatically after fertilisation (Wright et al., 1996). It was demonstrated that, in early stages of human embryonic cleavage, a recombination-based mechanism elongates telomeres (Liu et al., 2007b). From the blastocyst stage onwards, these elongated telomeres are then maintained by telomerase, without any further appreciable increase in length. Indeed, human embryonic stem (hES) cells isolated from the inner cell mass of the blastocyst invariably display high telomerase activity and long telomeres (Thomson et al., 1998, Reubinoff et al., 2000, Xu et al., 2001). Induced pluripotent stem (iPS) cells share almost all the functional properties of hES cells (Robinton and Daley, 2012). These cells can be derived from differentiated cells (telomerase negative cells) by forced expression of certain genes (e.g. OCT4, SOX2, c-MYC, and KLF4) or their proteins (Takahashi et al., 2007, Zhou et al., 2009b). During this transformation, the iPS cells acquire hTERT expression and telomerase activity equivalent to that in hES cells (Takahashi et al., 2007, Yu et al., 2007). The hES and iPS cell data indicate that higher telomerase expression is essential for the maintenance of stem cell characteristics, such as pluripotency, self-renewal and sell proliferation. However, fully functional iPS cells lacking fully functional telomerase activity due to haploinsufficiency were also successfully generated from Dyskeratosis Congenita patients, who lack telomerase activity due to a mutation in one allele of the hTERT or hTR genes (Agarwal et al., 2010, Batista et al., 2011). These iPS cells can then acquire telomerase expression in culture by upregulating the normal allele. These findings show that telomerase is not obligatory for the *formation* of iPS cells but is essential for their *maintenance* in culture.

Coming back to the cells in blastocyst, the telomerase activity in these cells gradually gets compartmentalised (**Figure II1.9**).

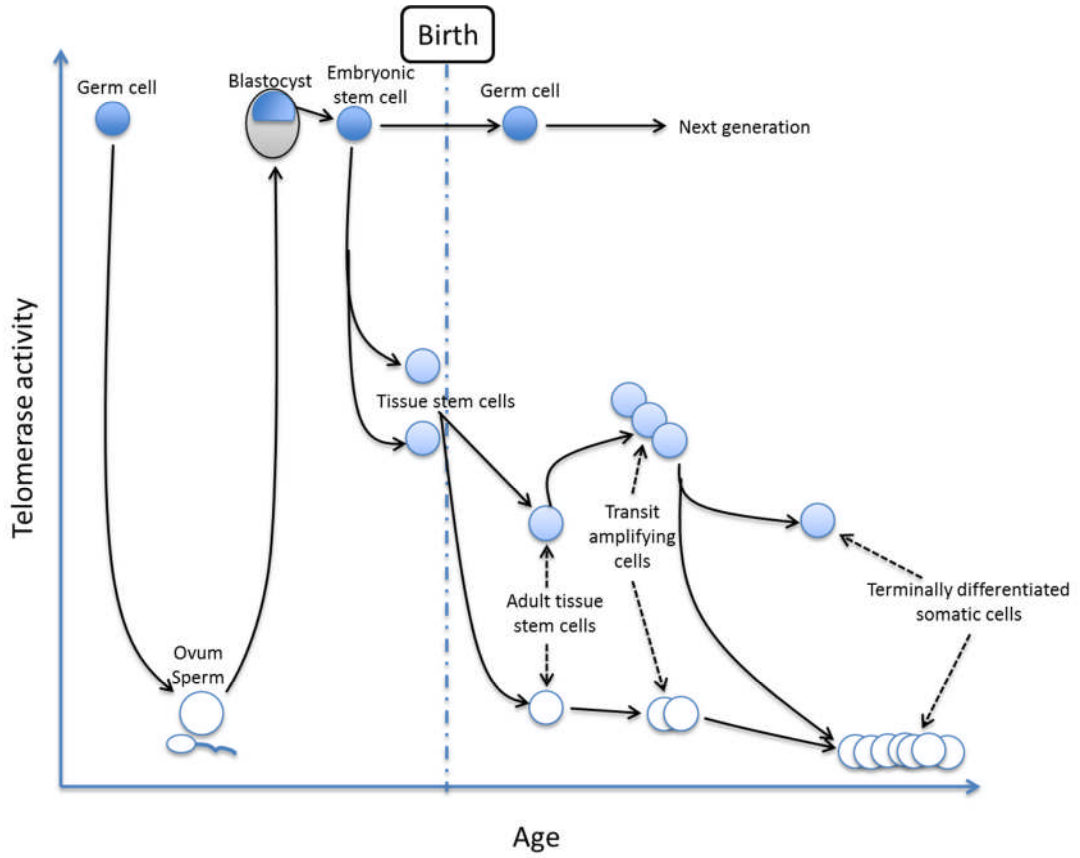


Figure II1.9: Telomerase activity during development. Unlike germ cells, mature ovum and sperm do not possess telomerase activity. The fertilised ovum rapidly acquires telomerase activity during the blastocyst stage. The embryonic stem cells (ESs), which have very high telomerase expression, differentiate into tissue-specific stem cells in the next 10-20 weeks of intrauterine life. These tissue stem cells have variable telomerase expression. After birth, only selected adult stem cells retain significant telomerase activity, which increases further on differentiation to transit amplifying cells. In an adult life, only very few terminally differentiated cells exhibit telomerase activity (e.g. T lymphocytes). All other somatic differentiated cells lack telomerase activity. Adapted from (Hiyama and Hiyama, 2007)

II1.3.2 Telomerase expression/activity in adult human tissues

Very few tissues exhibit detectable telomerase activity later than in embryonic life. At 16 weeks of intrauterine life, only liver and intestine display high telomerase activity. At the same time, lung, skin, kidney, and adrenal glands show marginally detectable telomerase activity; whereas, brain, bone and muscle tissues have lost

all detectable telomerase expression (Wright et al., 1996). This tissue-specific telomerase activity in foetal life is likely to be regulated by a combination of epigenetic modifications, direct transcriptional repression and alternative splicing of the hTERT subunit (Ulaner et al., 1998, Lopatina et al., 2003, Flores et al., 2006). The same mechanisms further restrict telomerase expression in adult life.

Initially, it was suggested that none of the normal human somatic tissues possess telomerase activity (Kim et al., 1994) and the only cells with telomerase activity were germ cells from testes and ovary (Wright et al., 1996). However, subsequent investigations identified detectable telomerase activity in: (i) normal peripheral lymphocytes and lymphocytes in bone marrow (Counter et al., 1995), (ii) the lower third of the normal human intestinal mucosa (Hiyama et al., 1996), (iii) the regenerative basal layer of the epidermis in 45% of samples (Harle-Bachor and Boukamp, 1996), (iv) proliferating cervical epithelium (Yasumoto et al., 1996), (v) 23% of normal oesophageal mucosa (Takubo et al., 1997), (vi) 95% of samples from the proliferative-phase of normal endometrium (Kyo et al., 1997), and (vii) haematopoietic cells in bone marrow (Yui et al., 1998). The puzzling observation with these studies was that detection of telomerase activity was always very unpredictable even in the same tissue. More notably, proliferation-dependent shortening of telomeres and senescence was observed in many of these tissues, most notably in intestinal mucosa (Hiyama et al., 1996), haematopoietic cells in marrow (Yui et al., 1998), peripheral lymphocytes (Wynn et al., 1998, Hodes et al., 2002) and in cultured fibroblasts (Masutomi et al., 2003) even in the presence of detectable telomerase activity. On the other hand, telomeres were maintained in cultured human mesenchymal stem cells even in the presence of negligible telomerase activity (Yanada et al., 2006). These observations led to the conclusion that, at least in adult human somatic tissue, the presence of detectable telomerase activity is neither sufficient nor necessary for unlimited or immortal cell replication

(Campisi, 1997). There are several propositions to explain these findings: (i) in cases where telomerase failed to maintain telomeres, telomerase may just extend proliferative potential by temporarily maintaining telomeres above critical length, (ii) extra-telomeric functions of telomerase are prominent in these tissues and, (iii) telomerase activity may be restricted to a specific small sub-population of cells.

II1.3.3 Telomerase expression/activity in adult stem cells

Subsequent studies showed that telomerase expression is restricted to such a small sub-population of cells where, both telomeric and extra-telomeric functions are being carried out by telomerase. Telomerase expression is associated with a stage of differentiation, whereby adult stem cells and terminally differentiated cells lacked telomerase activity, but the transit-amplifying cells displayed high telomerase activity. In the hematopoietic system, only the rapidly cycling transit amplifying cells exhibited high telomerase activity, which decreased upon terminal differentiation (Chiu et al., 1996, Yui et al., 1998, Brummendorf and Balabanov, 2006). Similar observations were also made in human epidermis, where telomerase activity was restricted to transit amplifying progenitor cells and was absent in stem cells and terminally differentiated cells (Bickenbach et al., 1998). It should be noted that both of these tissues have very high proliferation rate. Perhaps, telomerase activity is seen in adult cells only when they undergo proliferation at such a high rate. Surprisingly, there are insufficient telomerase expression studies in hierarchical sub-populations in human tissues (**Table II1.3**) to generalise these conclusions. However, it can be broadly concluded that human adult tissue stem cells possess minimal or no telomerase activity. This may be due to infrequent replication of these stem cells (Li and Bhatia, 2011). Once stem cells exit a quiescent state and differentiate into a rapidly proliferating, transit amplifying phenotype, they require more telomerase to maintain rapidly eroding telomeres. Probably for this reason, telomerase is also overexpressed in the majority of tumours.

Stem cell type	Telomerase	Telomeres	Reference
Embryonic	High	Maintained	(Thomson et al., 1998, Amit et al., 2000)
Mesenchymal	Low	Not maintained	(Yanada et al., 2006)
Haematopoietic	Detectable but low	Not maintained	(Brummendorf and Balabanov, 2006)
Epidermal	Absent	Not maintained	(Bickenbach et al., 1998)
Neuronal	Absent/low	Not maintained	(Wright et al., 2006, Varghese et al., 2008, Castelo-Branco et al., 2011)

Table II1.3: Telomerase activity in normal human adult stem cells. Table modified from (Hiyama and Hiyama, 2007)

II1.3.4 Telomerase expression/activity in cancer and cancer stem cells

The role of telomerase in cancer is ubiquitous and complex. About 80-90% of all cancers express high levels of telomerase, including cancers of prostate, breast, pancreas, liver, lung, brain, and intestine (Kim et al., 1994, Shay and Bacchetti, 1997). This acquisition of high levels of telomerase is a two-stage process (**Figure II1.10**) (Finkel et al., 2007). In an incipient tumour, cancer cells proliferate rapidly leading to telomere erosion. This telomere erosion activates cell cycle arrest/apoptosis/senescence pathways. In rare conditions, alternative lengthening of telomeres (ALT) mechanisms elongate telomeres and enable overt tumour growth (Heaphy et al., 2011). However in the majority of cases, aided by mutations in one or more cell cycle and DNA damage regulatory checkpoints, these cells acquire significant telomerase expression and the tumour reaches a homeostatic status (Artandi et al., 2000, Artandi and DePinho, 2010). At this point, telomerase actively promotes rapid tumour growth by maintaining shortened telomeres (not elongating them) and by activating cancer promoting pathways, such as WNT and c-MYC

pathways (as previously discussed in telomerase function section). However, it should be noted that telomerase is not a classical oncogene, as activation of telomerase alone does not cause oncogenic transformation (Jiang et al., 1999, Morales et al., 1999).

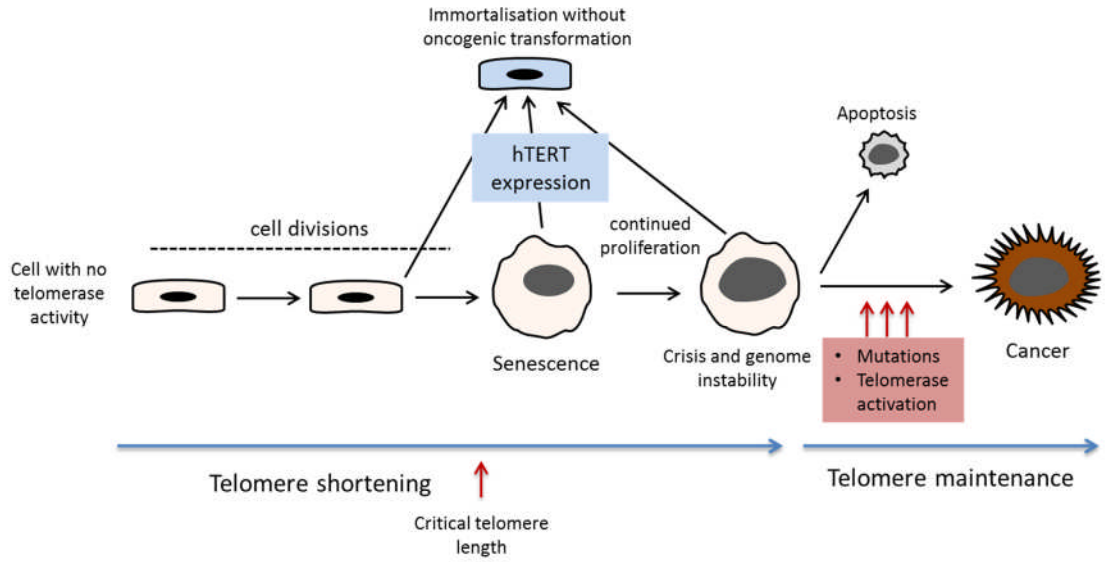


Figure II1.10: The role of telomerase in cell transformation. Adult somatic cells undergo telomere attrition with each division. When the critical telomere length is reached, senescence is triggered. In rare circumstances, aided by mutations in cell cycle and DNA damage repair checkpoints, cells continue to proliferate. This leads to genomic instability, due to the presence of unsustainably short and damaged telomeres. In most cases, this leads to apoptosis, but a rare clone may acquire telomerase activity and form a frank cancer. Cells can acquire "immortality" any time if telomerase is upregulated without the acquisition of mutations in key regulatory genes. Adapted from (Shay and Wright, 2006)

Apart from this umbrella role in all cancer cells, an additional layer of complexity for telomerase biology in cancer was identified recently. Several studies proposed that cancer stem cells (CSCs), which drive tumour progression, exhibit significantly higher telomerase expression than differentiated cancer cells (Joseph et al., 2010, Marian et al., 2010, Beck et al., 2011, Castelo-Branco et al., 2011, Serrano et al., 2011, Xu et al., 2011b). Telomerase can maintain this cancer stem-like cell state through telomere maintenance and activation of signalling pathways, such as

TERT-BRG1-NS/GNL3L transcriptional network and EGFR signalling (Shay and Wright, 2010, Beck et al., 2011, Okamoto et al., 2011). However, other studies suggested that stem-like cells in cancers exhibit very low telomerase expression, even in the same tissue where higher expression is also reported (Shervington et al., 2009). It is possible that the CSC telomerase expression status depends on individual tumour characteristics. Still, as most of these investigations rely heavily on 'stem cells' derived from cancer cell lines, they need further confirmation by analysis of actual human tumour derived fractionated cell sub-populations and robust in-vivo validation, to draw valid conclusions. Overall it can be concluded that, although telomerase is not an oncogene in a classical sense, it plays vital role in cancer progression.

II1.4 Telomerase in prostate and prostate cancer

Prostate epithelium is a very slowly cycling epithelium, where an adult epithelial cell divides, on average, every 200 days in the rat prostate (Isaacs and Coffey, 1989). The rate of cell division increases dramatically during benign or malignant transformation of the prostate epithelium. Ki-67 staining of human prostate tissue sections demonstrated about 2-3 fold increase in the proliferative index in BPH, while cancer displayed a 4-7 fold increase compared to normal tissue (Bubendorf et al., 1996, Kyprianou et al., 1996). Therefore it was hypothesised that telomerase, which is one of the principal regulators of cell proliferation, should be upregulated in prostate tumours. Indeed, investigations over the years consistently shown that about 80-90% of prostate cancers exhibit not only high telomerase activity but also short telomeres compared to normal prostate (**Table II1.4**).

Normal	Normal tissue adjacent to cancer	BPH	BPH tissue adjacent to cancer	PIN	Cancer	Reference
0/25	--	0/10	3/25	--	21/25 (84%)	(Sommerfeld et al., 1996)
--	5/20	--	--	--	14/30 (47%)	(Engelhardt et al., 1997)
--	--	2/13	--	--	14/18 (78%)	(Kallakury et al., 1997)
0/10	--	0/10	1/10	--	28/31 (90%	(Lin et al., 1997)
0/11	--	--	6/16	--	8/9 (89%)	(Scates et al., 1997)
--	2/19	--	--	--	18/22 (82%)	(Takahashi et al., 1997)
--	0/2	--	0/1	4/25	9/13 (69%)	(Koeneman et al., 1998)
0/124					39/42 (93%)	(Lin et al., 1998)
--	4/11	0/16	13/26	11/15	80/87 (92%)	(Zhang et al., 1998)
--	3/9	2/8	17/37	--	45/50 (90%)	(Wullich et al., 1999)
--	--	2/17	--	--	11/19 (58%)	(Wymenga et al., 2000)
0/18	--	--	--	--	17/18 (94%)	(Liu et al., 2001a)
1/8	--	--	--	--	32/35 (91%)	(Wang et al., 2001)

Table II1.4: Previous published studies, which assessed telomerase expression/ activity in patient-derived treatment naïve prostate tissue.

II1.4.1 Telomere and telomerase in BPH and PIN lesions

First, some key aspects that will help to comprehend the role of telomerase in prostate cancer were introduced by analysing telomerase expression in BPH and prostatic intraepithelial neoplasia (PIN). Telomerase expression has never been detected in normal prostate and is only rarely detected in BPH samples (Table II1.4). It is not clear why some BPH samples demonstrated telomerase expression and not others. Also, it is ironic that BPH does not display any telomerase

considering that the BPH is a long-standing hyperproliferative disorder with 2-3 fold higher proliferation index than normal prostate (Bubendorf et al., 1996, Kyprianou et al., 1996). This discrepancy in BPH telomerase expression may be partially explained by the observation that only 1-20% of nuclei from BPH tissue show hTERT expression by immunohistochemistry (Iczkowski et al., 2002). All of the cells with nuclear telomerase expression in these samples were basal cells. So, only a fraction of BPH basal cells express telomerase and therefore the content of such cells in a sample to be analysed may dictate the telomerase status of the sample as a whole. It is interesting to note that weak to moderate telomerase expression was found in about 10-15% of tissue with normal histology and about 10-50% of benign tissue adjacent to cancer (**Table II1.4**). Additionally, the average telomere length in BPH samples was equivalent to that of normal prostate epithelial telomere length; whereas the average telomere length in normal or BPH tissue specimens adjacent to cancer was significantly reduced and was similar to that in cancer specimens (Heaphy et al., 2010). These observations suggest that the histologically normal and BPH tissue specimens adjacent to cancer may contain cells with early oncogenic transformation and do not represent the pure normal or BPH tissue. Therefore, it is not surprising to find that PIN lesions, which are considered precursors for prostate cancer and often seen in a vicinity of it, show telomerase and a telomere pattern closer to cancer.

Initial studies revealed that about 65-75% of PIN specimens are telomerase positive by TRAP assay (Kim et al., 1994, Zhang et al., 1998). Later, using laser capture microdissection and TRAP assay, Koeneman et al. found telomerase in 4/25 (16%) of PIN lesions (Koeneman et al., 1998). This inconsistency continued when a more recent study, which found that hTERT was upregulated in almost all the high-grade PINs by in situ hybridisation (Bettendorf et al., 2003). These disagreements may well be due to the use of different techniques, which require variable sample

preparations. However, all the investigators who studied telomere length agreed that telomeres are shortened in the PIN lesions (Paradis et al., 1999, Meeker et al., 2002, Vukovic et al., 2003, Joshua et al., 2007, Heaphy et al., 2010). Vukovic et al. also showed that higher rate of telomere shortening was observed in PIN foci situated near (within 2 mm) adenocarcinoma (Vukovic et al., 2003). These findings provide additional strength for the belief that PIN is in fact a premalignant lesion that can develop into prostate cancer. Telomere shortening and telomerase upregulation in PIN lesions suggest that telomere shortening is an early event in the prostate carcinogenesis.

Telomere shortening on its own cannot cause cancer and it should be accompanied with cell cycle or DNA repair checkpoint abnormalities for frank malignant transformation. One such checkpoint could be 14-3-3sigma, which is downregulated in almost all prostate cancer foci compared to normal tissue (Lodygin et al., 2004). 14-3-3sigma is normally induced by P53 in response to DNA damage (Hermeking, 2003). The interesting point is that 14-3-3sigma downregulation (Lodygin et al., 2004) and telomere shortening in one of the studies (Meeker et al., 2002) was predominantly observed only in the PIN luminal cells. The luminal cells are terminally differentiated cells and therefore are least adaptable to cell proliferation seen during malignant transformation. For this reason, telomere-related abnormalities might be apparent earlier and in a more dramatic fashion in luminal cells, suggesting that the differentiation status of the cell should be considered while interpreting telomerase and cancer related results. However, it should be noted that no such luminal predisposition was reported in any of the other studies (Paradis et al., 1999, Vukovic et al., 2003, Joshua et al., 2007, Heaphy et al., 2010).

II1.4.2 Activation of telomerase in PIN lesions with short telomeres

The shortening of telomeres in PIN and morphologically normal surrounding tissue may represent areas of risk for prostate carcinogenesis. An inverse correlation

between the length of telomeres in normal-appearing prostate tissue adjacent to a tumour, and a 72-month recurrence-free survival indicate that telomere shortening precedes telomerase activation and frank cancer formation in the prostate (Fordyce et al., 2005). Both genetic instability and end-to-end chromosome fusions resulting from short telomeres may then contribute towards prostate oncogenesis. Once natural selection selects the clones with favourable mutations providing robust malignancy potential, telomerase is overexpressed in the SC clones (Campbell, 2012). So in effect, telomerase stabilises the genomic crisis state generated by telomere shortening to produce tumour homeostasis. A recent report by Ding et al. elucidates this phenomenon in a prostate cancer mouse model (Ding et al., 2012). They generated P53/PTEN null mice with inducible mTERT to investigate the role of telomerase activation after telomere shortening. The P53/PTEN null mice invariably develop invasive prostate adenocarcinoma by 24 weeks of age in the presence of telomerase. The P53/PTEN null mice lacking telomerase could only develop much smaller, less invasive and less aggressive tumours. The tumours lacking telomerase possess irreparably damaged short telomeres with non-sustainable chromosomal fusions. In such cases, the disadvantages of continued genomic instability outweigh any potential gains from further genomic evolution. When telomerase is re-expressed in these tumours, they readily transform into a highly aggressive, invasive phenotype. The re-expression of hTERT not only restores telomeres and hence, genomic stability but also contributes towards carcinogenesis via non-telomere dependent activation of TGFβ-SMAD4 pathway. The observation that P53/PTEN mutations are necessary for prostate carcinogenesis in addition to telomerase overexpression, along with similar observations using primary human prostate epithelial cells (Burger et al., 1998), reaffirm that prostate tumorigenesis is a multistep process and telomerase activation alone is not sufficient for malignant transformation of prostate.

II1.4.3 Telomerase in prostate cancer

In over 80% of prostate cancers, overexpression of telomerase was detected. Owing to the high telomerase expression in prostate cancer, several studies investigated telomerase as a marker for prostate cancer diagnosis, prognosis or as a therapeutic target. In order to increase the ease of detection in a clinical setting and to improve sensitivity and specificity of the detection, several methods of telomerase detection were assessed. First, the TRAP assay was performed on tumour needle biopsy samples (Sommerfeld et al., 1996, Kallakury et al., 1997, Takahashi et al., 1997). Later, when it was noted that the tumour biopsy cancer content varied significantly and that this affected telomerase levels drastically (Engelhardt et al., 1997), targeted laser capture microdisection was employed to maximise tumour content of the tissue (Liu et al., 2001a). In addition, tissue material obtained from touch imprinted biopsies (Chieco et al., 2001), fine needle aspiration cytology of tumours (Wang et al., 2002), cells collected after prostatic massage (Vicentini et al., 2004), exfoliated cells from urine (Botchkina et al., 2005), and even circulating plasma/serum (Dasi et al., 2006, Dalle Carbonare et al., 2011, March-Villalba et al., 2012) were all investigated to improve the usability of bedside telomerase detection. The TRAP assay still remains the gold-standard for telomerase detection, but techniques such as qPCR (Liu et al., 2001a, Botchkina et al., 2005), PCR-ELISA (Wang et al., 2002), immunohistochemistry (Iczkowski et al., 2002), in-situ hybridisation (Kamradt et al., 2003) were also employed with variable success. Paradoxically in all these studies, any association between telomerase levels and prostate cancer prognosis was mixed. A positive correlation between telomerase and tumour aggressiveness was noted in at least 5 studies (Lin et al., 1998, Wullich et al., 1999, Wymenga et al., 2000, Wang et al., 2001, Athanassiadou et al., 2003), but an almost equal number of studies could not find such correlation (Kallakury et al., 1997, Zhang et al., 1998, Bettendorf et al., 2003, Kamradt et al.,

2003, Pfitzenmaier et al., 2006). There could be several explanations for this disagreement. First, some investigations, such as Wullich et al., showed that telomerase expression is heterogeneous in prostate cancers, especially in cancers with Gleason grade less than 7 (Wullich et al., 1999). The intra-tumour heterogeneity and high telomerase in PIN lesions may lead to false positive or false negative telomerase detection results, and could have partly contributed to the above variable correlations. Secondly, the relative content of inflammatory infiltrate, which has detectable telomerase activity (Liu et al., 1999), could confound the correlation between telomerase expression and indicators of tumour prognosis. Isolation of pure and homogeneous primary prostate cancer cells seems probably the best way to obtain reliable and representative results. Nevertheless, high telomerase expression in prostate cancer attracted multiple attempts to discover an efficient telomerase inhibitor for the treatment of the prostate cancer.

II1.5 Targeting telomerase as a therapy for prostate cancer

Therapeutic targeting of telomerase in prostate cancer has been at the forefront of the research associated. From steroid hormones to natural molecules and oligonucleotides to targeted virus-mediated therapy; many options have been tested in last few years (Table II1.5). Attempts were made to link androgen ablation therapy and estrogen manipulation with telomerase inhibition, but it resulted in equivocal results. Half of the studies indicated a positive relationship between androgen/estrogen and telomerase expression, while the other half showed the opposite. The same was true for plant isoflavone, Genistein. On the other hand significant inhibition of telomerase activity was achieved in predominantly prostate cell line models by decreasing hTERT/hTR transcription or via direct enzymatic inhibition (**Table II1.5**). A synthetic compound imetelstat, which inhibits telomerase enzyme activity, is one of the most promising current telomerase inhibitors. It is in

phase-I/II clinical trials for the management of other cancers, such as breast cancer and multiple myeloma and was also proposed to be effective in prostate cancer models (Asai et al., 2003, Marian et al., 2010, Roth et al., 2010). However, none of these agents have been registered for clinical trials for the management of prostate cancer so far. Because most of these studies were performed in cell lines, the specificity and actual efficacy of these agents in patients is completely unknown. The precise direct mechanism of action for the majority of proposed interventions also remains to be evaluated. Therefore, the use of telomerase inhibitors for the management of prostate cancers is still speculative.

Agent	**Model**	**Proposed mechanism**	**Reference**
Activation of telomerase			
Zinc	DU145	?	(Nemoto et al., 2000)
Insulin-like growth factor-1	PC3, DU145, LAPC-4	AKT-mediated increase in hTERT transcription	(Wetterau et al., 2003)
Inhibition of telomerase			
Vitamin D3	LNCaP	? Differentiation	(Hisatake et al., 1999)
Nerve growth factor	DU145 and PC3	?	(Sigala et al., 1999)
2-5A-anti-hTR: hTR small molecule inhibitor	PC3, DU145, and PC3 xenografts	Inhibition of hTR	(Kondo et al., 2000)
9-nitrocamptothecin	DU145 xenografts	Inhibition of hTERT, c-MYC and BCL2	(Chatterjee et al., 2000)
Antisense phosphorothioate oligonucleotides (PTO)	DU145	Inhibition of hTERT transcription	(Schindler et al., 2001)
Dominant negative hTERT	LNCaP, DU-145, and PC3 xenografts	Inhibition of hTERT transcription	(Guo et al., 2001)
Trichostatin A (inhibitor of Histone deacetylases)	PC3, LNCaP	? No direct link	(Suenaga et al., 2002)

Vitamin D3 + 9-cis-retinoic acid	PC3	Inhibition of hTERT transcription	(Ikeda et al., 2003)
Silibinin	LNCaP	Inhibition of hTERT transcription	(Thelen et al., 2004)
2'-O-methyl-RNA phosphorothioate oligonucleotides	DU145	Decrease in full-length hTERT and concomitant increase in dominant negative splice variant	(Brambilla et al., 2004)
Anti-sense nucleotide for hTR	PC3	Inhibition of hTR	(Shariftabrizi et al., 2005)
Small molecule antisense oligonucleotide-based inhibitor (ISIS 125628) alone or in various combinations	C4-2/C4–2B	hTR template antagonist	(Canales et al., 2006)
OBP-301 (Telomelysin, a telomerase-specific replication-competent adenovirus with hTERT promoter)	LNCaP xenografts	Direct lysis of telomerase expressing prostate cancer cells.	(Huang et al., 2008)
Imetelstat	DU145, C4-2 and LNCaP	hTR template antagonist	(Asai et al., 2003, Marian et al., 2010)
Ambiguous results			
Androgen	Rat prostate epithelium	Inhibition of telomerase activity	(Meeker et al., 1996)
	LNCaP		(Soda et al., 2000)
	PC3 and PC3-AR cells		(Moehren et al., 2008)
	LNCaP and CWR22 xenograft	Enhancement of telomerase activity	(Guo et al., 2003)
	Prostate cancer patients		(Iczkowski et al., 2004)
	LNCaP		(Thelen et al., 2004)
	LNCaP		(Geier et al., 2010)
Estrogen	LNCaP	Inhibition of telomerase activity	(Stettner et al., 2007)
	LNCaP		(Geier et al., 2010)
	Human normal	Enhancement of	(Nanni et al.,

	prostate epithelial cells, fresh explants from benign prostatic hyperplasia, prostate cancer explants, and prostate cell lines	telomerase activity	2002)
	PC3		(Chen et al., 2009)
Genistein	DU145 and LNCaP	Inhibition of hTERT, c-MYC and upregulation of P21	(Ouchi et al., 2005)
		Repression of hTERT transcriptional activity via c-MYC and posttranslational modification of hTERT via AKT	(Jagadeesh et al., 2006)
		STAT3 mediated hTERT activation	(Chau et al., 2007)

Table II1.5: Chemical agents used to modify telomerase expression/activity in prostate.

With the emerging evidence for the role of cancer stem cells/tumour initiating cells in progression and relapse of multiple cancer, the investigation of telomerase expression and activity in this specific cell type has become instrumental (Maitland and Collins, 2008b, Visvader and Lindeman, 2008, Clevers, 2011). It has been proposed that cancer stem cells are primarily responsible for prostate cancer relapse after any treatment, for instance androgen ablation therapy for prostate cancer (Rizzo et al., 2005, Collins and Maitland, 2009, Qin et al., 2012). Therefore, it is critical to assess the possibility of telomerase targeting agents specifically on the survival of these cells. Previous studies have suggested that hTR mRNA is expressed in the normal basal cells, where normal stem cells also reside (Paradis et al., 1999). However, hTR is ubiquitously expressed in most of the human tissues and cannot be taken as a surrogate for telomerase activity. Two recent

investigations did attempt to analyse telomerase expression in prostate cancer stem cells, but their findings do not match with each other (Marian et al., 2010, Xu et al., 2011b). As of now, telomerase biology in normal and cancer prostate stem cells remains to be explored in detail.

SECTION II: 2. Aims and objectives

Telomerase is overexpressed in majority (>80%) prostate cancers and is undetectable in normal or BPH tissue (Zhang et al., 1998). Data obtained from human cell lines and mouse models of prostate cancer further demonstrate that telomerase is beneficial for prostate cancer growth, not only for its telomere maintenance function, but also for its non-telomeric functions. With this data, telomerase represents a valid target for prostate cancer management. Two recent studies endeavour to prove that telomerase inhibition could also abolish the CSC population in prostate cancer (Marian et al., 2010, Xu et al., 2011b). However, there are still several important questions:

1. Do normal stem cells express telomerase?
2. As BPH is a hyperproliferative disorder and telomerase is a principal regulator of cell proliferation, why does BPH not display 'any' telomerase reactivity?
3. What is the telomerase expression status in enriched primary prostate epithelial hierarchical cancer populations (including CSCs)?
4. Can telomerase inhibition abolish CSCs and/or their tumour inducing function?
5. What is the telomerase expression status in castration resistant prostate cancer?
6. Why do telomerase inhibition therapies fail to abolish tumours completely in some cancer models?

To answer these questions definitively in relation with prostate cancer, we assessed expression/activity and functional effects of telomerase in primary pure and homogeneous primary prostate epithelial sub-populations enriched from normal, BPH, treatment naïve cancer, and castration resistant prostate cancer patients.

SECTION II: 3. Results

II3.1 Assessment of telomerase expression in prostate epithelium

II3.1.1 Determination of hTERT mRNA levels prostate cell lines

In order to have a comparison reference point and validate the experimental set-up by confirming previously published telomerase expression results (Marian and Shay, 2009), mRNA levels of the hTERT subunit of the telomerase enzyme was measured by qRT-PCR using a TaqMan probe in prostate epithelial cell lines (**Figure II3.1**). Normal and benign prostate cell lines (PNT2c2, PNT1a, and BPH1) showed minimal hTERT expression compared to cancer cell lines (DU145, LNCaP, and VCaP). The benign (RC-165N/hTERT) and malignant (RC-92a/hTERT) prostate cell lines, which had been immortalised using hTERT transfection understandably, displayed significantly higher hTERT expression. Of note, the P4E6 cancer cell line, which was derived from very early stage prostate cancer (Maitland et al., 2001) and the highly tumorigenic PC3 cell line (Zhang et al., 2003b), exhibited hTERT expression similar to normal/benign cell lines. In summary, hTERT is overexpressed in the majority of prostate cancer cell lines as compared to normal/benign cell lines.

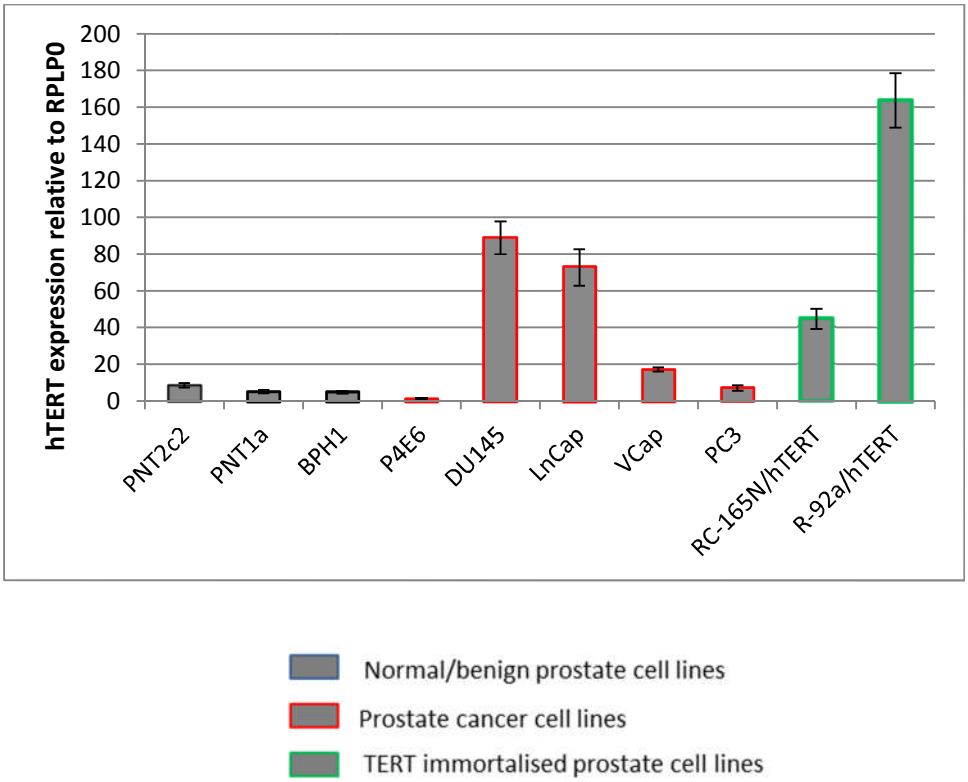


Figure II3.1: hTERT mRNA levels in prostate epithelial cell lines. Expression of hTERT subunit of telomerase was determined in prostate epithelial cell lines by qRT-PCR analysis. Expression was normalised to internal control RPLP0 and plotted relative P4E6 hTERT expression.

II3.1.2 Determination of hTERT mRNA levels in patient-derived primary prostate epithelial cultures

Subsequent analysis of telomerase expression was performed in epithelial cultures (primary cultures) obtained from patient-derived prostate samples for two main reasons: (i) primary cultures represent patient prostate tumours more closely than cell lines (Peehl, 2005), and (ii) long-term cell line maintenance in culture alter telomerase expression in cell lines, which may be very different from that in the tissue of origin (Lin et al., 1997, Soda et al., 2000). For this analysis, the cell line phenotypically closest to the primary epithelial cultures, P4E6, was chosen as a reference point (Maitland et al., 2001). The cell line with one of the highest hTERT expression (RC-165N/hTERT) was used for additional comparisons.

The telomerase expression pattern was determined by analysing hTERT mRNA levels in cultures derived from BPH (n=3), cancer (n=3), and castration resistant prostate cancer samples (CR-PCa, n=3) at passage 2 in culture (**Figure II3.2**). Expression in one of the BPH cultures was undetectable, while the rest had significantly lower expression than the cancer cultures. The highest hTERT expression was observed in CR-PCa derived cultures, which was significantly higher than in the treatment naïve cancer cultures. Comparison of hTERT expression in primary cultures with reference to telomerase expression in the reference cell lines (P4E6 and RC-165/hTERT) revealed that all the primary cultures all expressed significantly less hTERT than the routinely used cell lines (P4E6 cell line had the lowest hTERT mRNA levels among all the cell lines tested, **Figure II3.1**). In brief, CR-PCa and cancer cultures displayed significantly higher hTERT expression than BPH cultures.

These observations demonstrated that the expression pattern of intact tissue samples (which is composed of basal and luminal cells) was preserved in primary

cultures (which contain only basal cells) (Richardson et al., 2004, Collins et al., 2005). However, prostate epithelial tissues were shown to be composed of populations with distinct proliferation and self-renewal properties (Collins et al., 2001, Collins et al., 2005). As telomerase was predicted to heavily influence both of these properties (Shay and Wright, 2010), we hypothesised that telomerase would be differentially expressed in these populations.

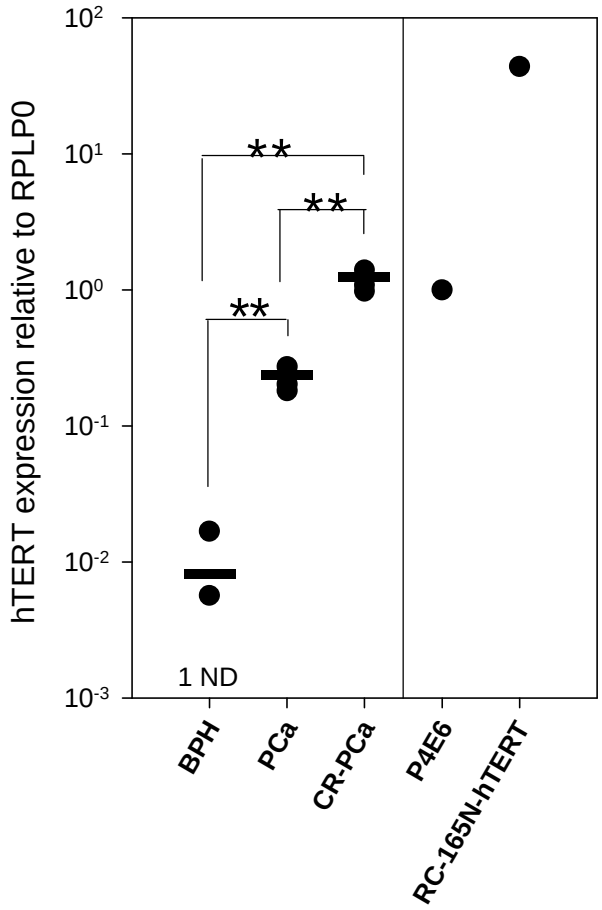


Figure II3.2: hTERT mRNA levels in primary prostate epithelial cultures. Expression of hTERT subunit of telomerase was determined in BPH (n=3), cancer (n=3) and CR-PCa (n=3) cultures (passage 2) by qRT-PCR analysis. Expression was normalised to internal control RPLP0 and plotted relative P4E6 hTERT expression. The horizontal bars indicate the mean expression. [BPH: benign prostatic hyperplasia, PCa: prostate cancer, CR-PCa: castration resistant prostate cancer, **p<0.01- unpaired Student's t-test, ND: not-detected]

II3.1.3 Determination of hTERT mRNA levels in individual sub-populations enriched from patient-derived primary prostate epithelial cultures

Telomerase is known to influence both proliferation potential and self-renewal, either through telomere length maintenance or through the interactions with signalling pathways, such as WNT and NOTCH (Park et al., 2009, Shay and Wright, 2010). Epithelial hierarchical sub-populations enriched from the prostate display significant variations in these properties, for example stem cells exhibit significantly more proliferative and self-renewal potential than differentiated committed basal cells (Richardson et al., 2004, Collins et al., 2005, Garraway et al., 2010, Qin et al., 2012) . In order to check whether telomerase expression informed about active self-renewal and proliferative potential, the hTERT mRNA levels status was measured in the hierarchical sub-populations enriched from the primary normal, benign and cancer samples.

We found that none of the normal primary epithelial sub-populations had detectable hTERT expression (**Figure II3.3A**). The undifferentiated sub-populations derived from BPH cultures (SC and TA) expressed significantly higher hTERT mRNA than committed basal cells (Figure II3.3A). The BPH-derived CB sub-population showed undetectable hTERT expression in 4/5 cases. In cancer samples, hTERT expression was undetectable in stem cells, but was significantly higher in TA and CB cells. Finally, the hTERT expression in luminal cells was undetectable in 6/7 non-cancer cases but was always present in cancer derived luminal cells (**Figure II3.3B**)

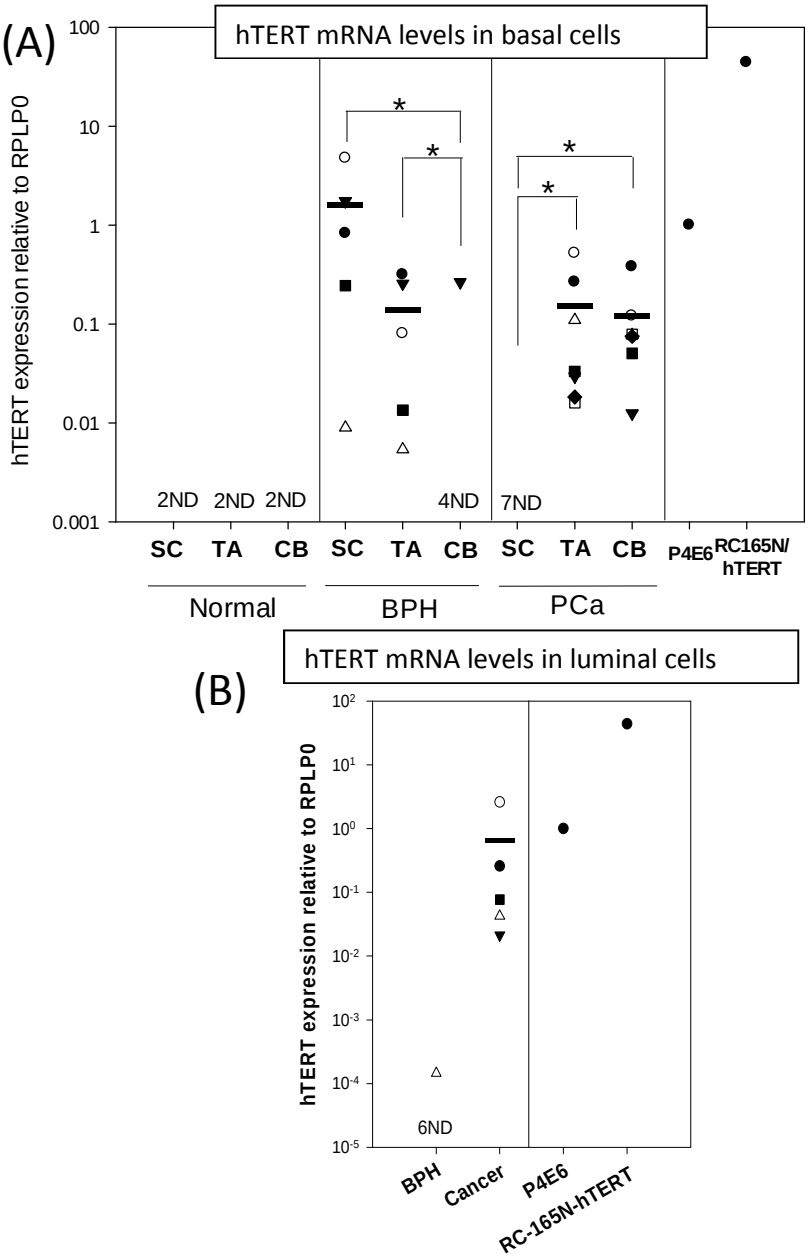


Figure II3.3: Telomerase mRNA levels in sub-populations enriched from primary prostate epithelial cultures. **A**: Expression of hTERT subunit of telomerase was determined in individual cell sub-populations derived from cultured normal, benign, and cancerous primary prostate epithelial cells (passage 2) by qRT-PCR analysis. **B**: Expression of hTERT subunit of telomerase in luminal cells enriched from benign and cancerous prostate tissue before culture. Expression was normalised to the internal control RPLP0 and plotted relative P4E6 hTERT expression. The horizontal bars indicate the mean expression. [BPH: benign prostatic hyperplasia, PCa: prostate cancer, SC: stem cells, TA: transit amplifying cells, CB: committed basal cells, LC: luminal cells, *p<0.05 - paired two-tailed t-test, ND: not-detected]

II3.2 Assessment of telomerase activity in prostate epithelium

The hTERT subunit is the rate-limiting catalytic sub-unit of the telomerase enzyme. In the majority of the cases, hTERT expression status accurately informs about telomerase activity of most samples (Ito et al., 1998, Wu et al., 1999, Kirkpatrick et al., 2003, Li et al., 2003). However, the telomerase enzyme needs assistance from at least 32 other proteins while performing its function and post-translational hTERT modifications may inhibit its functionalities (Aisner et al., 2002, Kim et al., 2005, Cohen et al., 2007). Therefore, hTERT mRNA levels alone may not necessarily represent telomerase activity in all circumstances (Ramakrishnan et al., 1998, Snijders et al., 1998, Tahara et al., 1999). In order to measure direct telomerase functionality, we determined telomerase activity using an ultra-sensitive (can measure telomerase activity in about 10-100 cells accurately – necessary for determining activity in a very small number of cells such as stem cells) mini-TRAP assay (Kim et al., 1994, Herbert et al., 2006). Owing to its technical simplicity, the newly developed qPCR based mini-TRAP assay protocol was preferred to widely utilised gel-based TRAP assay (Herbert et al., 2006).

It was noted that, in BPH (n=5), telomerase activity in undifferentiated cells (SC and TA cells) was significantly higher than in differentiated CB cells (**Figure II3.4**). In cancer (n=6), the undifferentiated SCs exhibited extremely low to non-detectable telomerase activity, while TA and CB cells displayed significantly higher telomerase activity. In contrast, the spread of the data in BPH-derived subpopulations was minimal, while it was much bigger in cancer. This spread reflected higher heterogeneity in cancer than BPH. The wider spread in cancer-derived sub-populations (especially TA and CB cells) probably signifies the heterogeneous nature and patient specific variations in prostate cancer.

The synopsis is that, in prostate epithelial cultures, hTERT qRT-PCR expression reflected the telomerase activity with fair accuracy. Both of these measurements were quantitatively comparable, and therefore for further experiments, only hTERT qRT-PCR expression was measured and taken as a surrogate for telomerase activity.

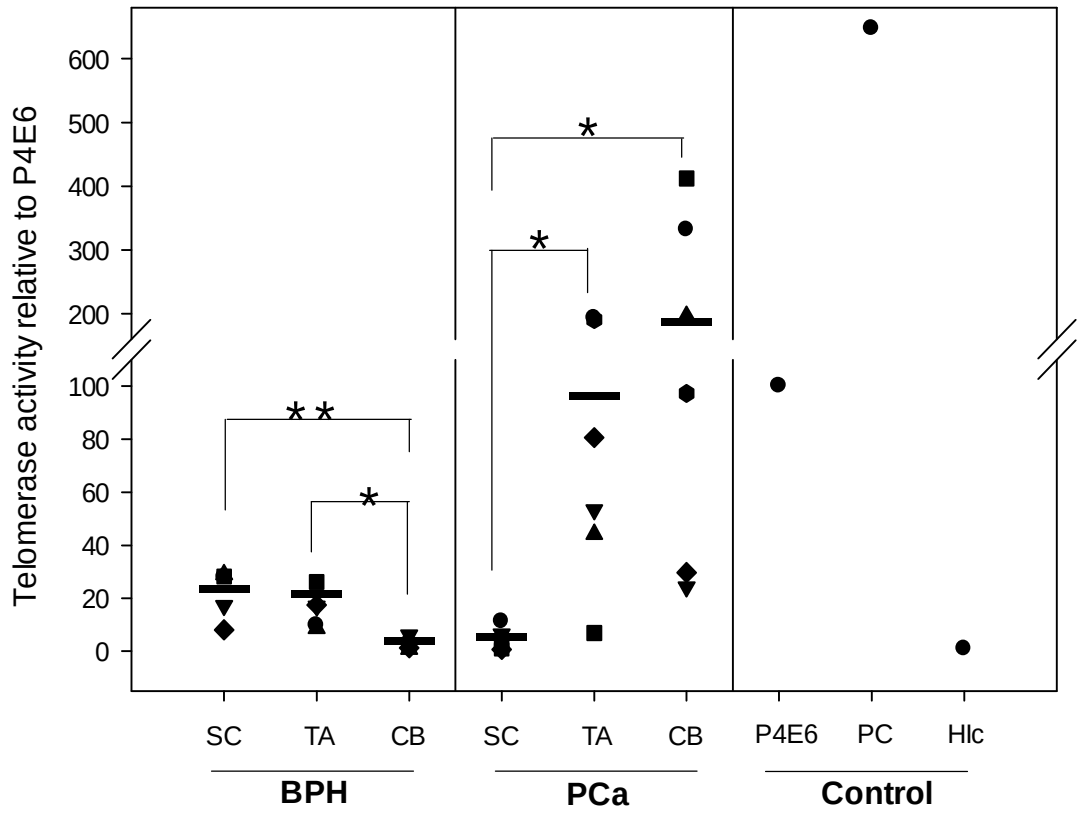


Figure II3.4: Telomerase enzymatic activity in sub-populations enriched from primary prostate epithelial cultures. Endogenous telomerase activity was determined in individual cell sub-populations derived from cultured benign (n=5) and cancerous (n=6) primary prostate epithelial cells (passage 2) by qPCR using mini-TRAP assay. Expression was plotted relative P4E6 telomerase activity and the horizontal bars indicate mean expression. [BPH: benign prostatic hyperplasia, PCa: prostate cancer, SC: stem cells, TA: transit amplifying cells, CB: committed basal cells, *p<0.05, **p<0.01 - paired two-tailed t-test, PC: positive control in the kit, HIc: heat inactivated control, ND: not-detected]

II3.3 Assessment of telomerase expression under cellular stress

One of the most intriguing results of the last two experiments was that cancer stem cells (CSCs) did not display telomerase expression and activity. As it is possible that the microenvironment may also influence the telomerase expression in CSCs/CSC-like cells (and other sub-populations), telomerase expression was next measured in epithelial sub-populations enriched from CR-PCa and xenografts. It is very likely that these two conditions assert severe pro-proliferative stress on CSCs, in addition to challenges for adjusting to the vastly modified microenvironment.

We found that hTERT mRNA levels in all of the sub-populations from CR-PCa and xenografts (**Figure II3.5**) was higher than treatment naive cancers (compare to **Figure II3.3**). The undifferentiated SC population from CR-PCa and $CD133^{+}$ sub-population from xenografts also exhibited hTERT expression of a similar magnitude as other sub-populations. This was in stark contrast with SC population hTERT expression in SC populations enriched from treatment naïve samples (compare to **Figure II3.3**).

This suggested that the SC population can acquire hTERT expression and the telomerase expression is at least partly dependent on the pathological status and tumour microenvironment in prostate epithelial sub-populations.

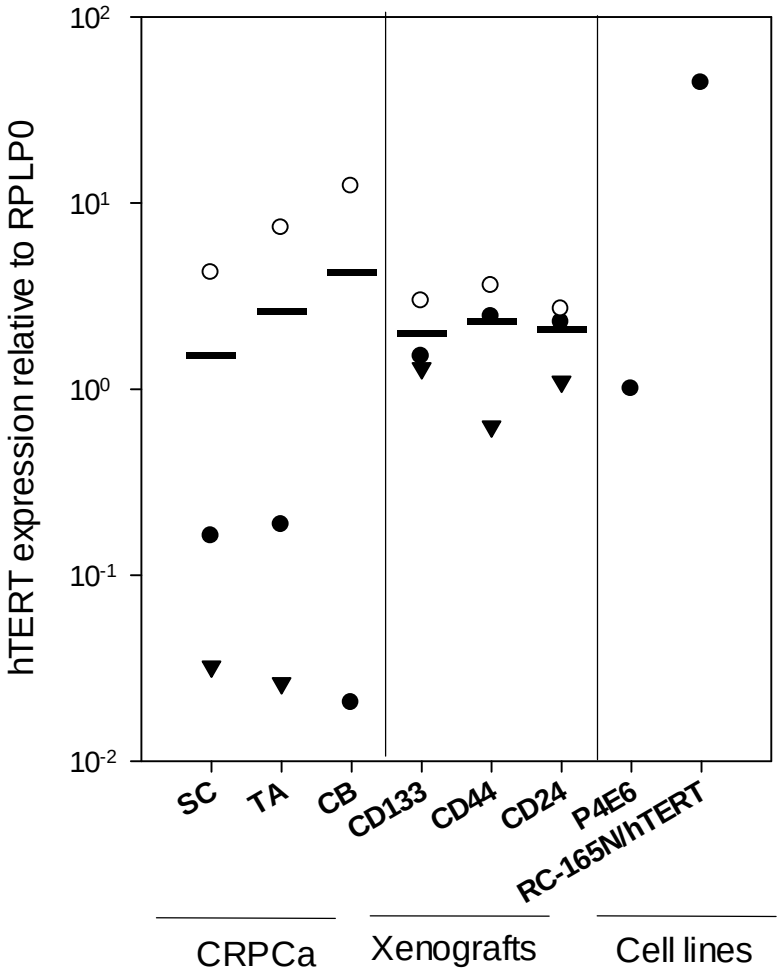


Figure II3.5: Telomerase mRNA levels in sub-populations enriched from castration resistant prostate cancers (CRPCa) and xenografts. Expression of hTERT subunit of telomerase was determined in individual cell sub-populations derived from cultured CRPCa primary epithelial cells (n=3, passage 2) and mouse lineage depleted prostate xenograft tissue (n=3, 2 of them were derived from CRPCa primary tumours) by qRT-PCR analysis. Expression was normalised to the internal control RPLP0 and plotted relative P4E6 hTERT expression. The horizontal bars indicate the mean expression. [SC: stem cells, TA: transit amplifying cells, CB: committed basal cells]

II3.4 Functional assessments after telomerase inhibition

So far, it was clear that the telomerase is differentially expressed in primary prostate epithelial sub-populations. Previous investigations in other laboratories also showed that telomerase could regulate stem cell self-renewal, in addition to the regulation of cell proliferation (Chapman et al., 2008, Kirwan and Dokal, 2009, Pech and Artandi, 2011). Considering the fact that telomerase is critical for the maintenance of proliferation, together with the above two findings, we hypothesized that telomerase could be essential for the maintenance and cell fate determination of prostate epithelial sub-populations. A functional assessment that specifically probes the role of telomerase inhibition in primary prostate cells was therefore essential to identify cues regarding the therapeutic potential of telomerase inhibition for the management of prostate cancer (and indeed BPH). Therefore, to investigate the functional role of the telomerase in BPH and cancer cultures, loss-of-function studies were performed.

For telomerase loss of function studies, hTERT expression was inhibited using directed and specific siRNA. This approach had following advantages: (i) it was a validated, efficient, and specific method, (ii) it was possible to reach high transfection efficiency (due to the small size of the siRNA) without compromising the viability of the primary samples, and (iii) therefore did not require selection of the transfected cells. Selection processes were avoided as they could indeed affect the cell identity and their telomerase expression due to cellular stress during selection. Alternative approaches were the use of small molecule telomerase inhibitors or short hairpin RNAs (shRNAs). But they were not readily available and their specificity and effects were not validated. The shRNA transfection efficiency of primary samples was also limited at the time (Thesis of S. Jacoby, 2011).

II3.4.1 Validation of siRNA mediated telomerase inhibition

The efficiency of siRNA-mediated telomerase inhibition was assessed by analysing hTERT mRNA levels and telomerase activity 3 and 7 days after transfection. As unfractionated BPH cultures have minimal or no telomerase activity, in order to have a precise measurement of telomerase inhibition, BPH progenitor cells (SC and TA cells) were selected after siRNA treatment and used for this experiment, while cancer cultures were unfractionated. At 50nM concentration, telomerase activity, as determined by mini-TRAP assay, was decreased by about 70% in BPH and cancer cells (**Figure II3.6**). Based on this information, BPH and cancer-derived cultures were transfected with 50nM of siRNA and functional effects were assessed after a week of transfection using multiple cell fate assays (viable cell count, senescence, proliferation, apoptosis, and colony forming efficiency assay).

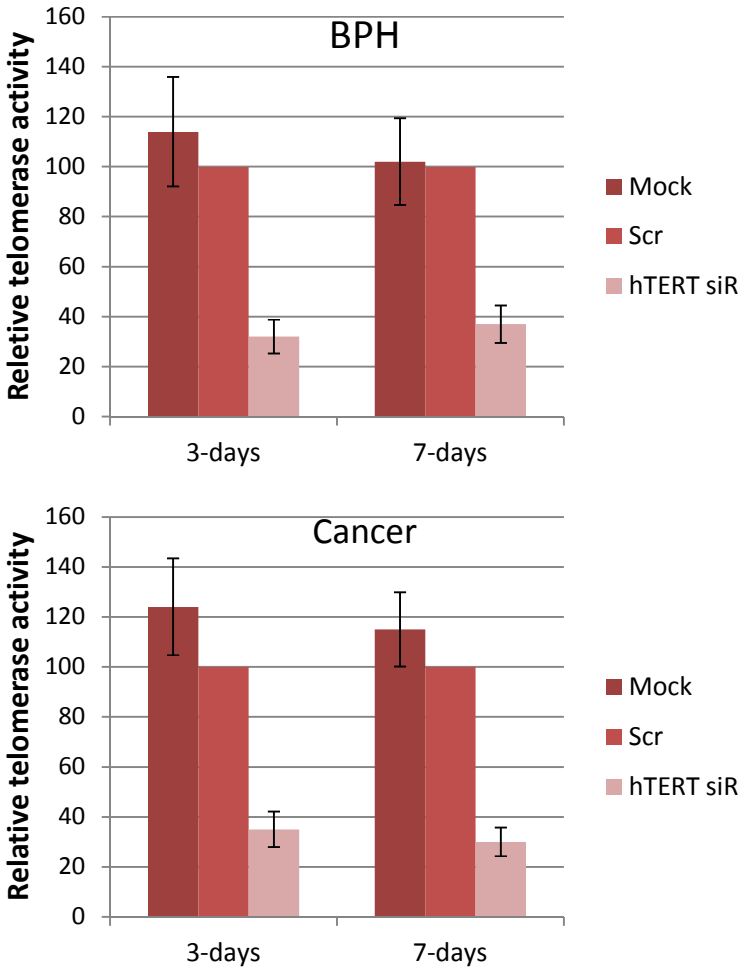


Figure II3.6: Telomerase inhibition by hTERT directed siRNA. BPH-derived progenitor cells (n=3) and cancer-derived cultures were transfected with 50nM scrambled/hTERT siRNA. The telomerase activity on day 3 and 7 was detected by qRT-PCR based TRAP assay.

II3.4.2 Effect of telomerase inhibition on cell survival

Inhibition of the hTERT sub-unit of telomerase is known to inhibit cell survival in multiple cell types, including prostate cell lines (Folini et al., 2005). To check whether the same is true for primary prostate epithelial samples, viable cells were counted over a period of 7 days after hTERT siRNA mediated telomerase inhibition (**Figure II3.7**). Since the cancer-derived cultures used in the experiment were growing at a rapid rate, this experiment was started with 25,000 cancer derived epithelial cells, whereas for BPH, 40,000 cells were plated in each well of a 6-well collagen I coated plate. This was necessary to minimise passaging of cells during the experiment. The analysis by Trypan blue dye exclusion test showed that the viable cell count in BPH derived cultures reduced significantly by day 5 and 7 as compared to scrambled and mock transfected controls. Although a reduction in viable cell count was also seen in cancer samples after 5 and 7 days of hTERT inhibition, the magnitude of the reduction was not as marked as in BPH and was statistically insignificant. Inhibition of telomerase is known to induce a reduction in viable cell count predominantly via one or more of the following mechanisms: induction of senescence, reduction in cell proliferation and induction of apoptosis (Shay and Wright, 2006, Ouellette et al., 2011). Therefore, these parameters were investigated in further analysis.

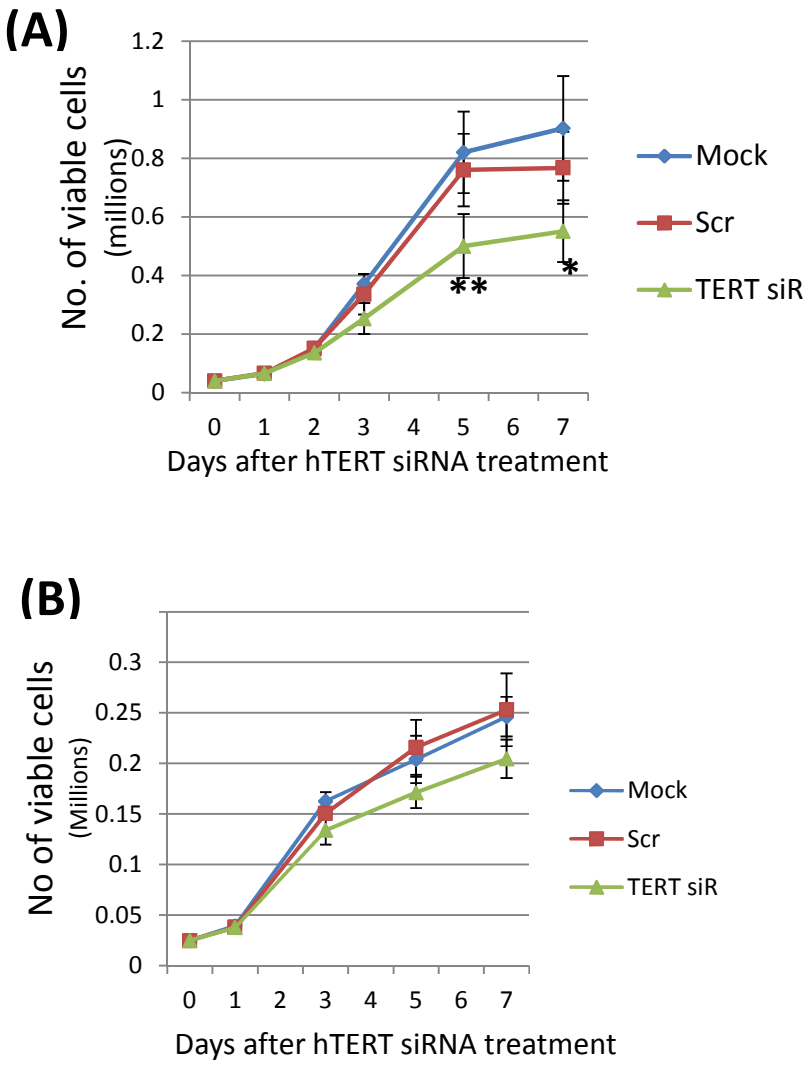


Figure II3.7: Cell survival analysis after telomerase (hTERT) inhibition. Primary epithelial cultures derived from BPH (**A**, n=5) and prostate cancer (**B**, n=3) tissues were grown till passage 2 and were transfected with 50nM of hTERT directed siRNA (TERT siR) and scrambled siRNA (Scr). Viable cell count was measured by Trypan blue exclusion test on specified time points. Initial cells plated without STOs: BPH cultures: 40,000 and cancer cultures: 25,000. *p<0.05, **p<0.01 - paired two-tailed t-test

II3.4.3 Effect of telomerase inhibition on cellular senescence

Induction of replicative senescence is one of the prime effects of telomerase depletion (Asai et al., 2003). This mechanism constitutes the most important rationale for using telomerase inhibition in cancer management. Upon induction of senescence, endogenous lysosomal β-galactosidase is overexpressed, accumulates and can be specifically detected at acidic pH (Campisi, 1997, Shay and Wright, 2005). Although this is a semi-quantitative method, it is the most commonly used and accepted method for the detection of senescence. After 7 days of hTERT or scrambled siRNA transfection, cells were fixed and stained for β-galactosidase overnight. The blue/green stained cells were divided into intensely stained cells and weakly stained cells and counted manually to quantify senescence. Application of this method revealed a significant increase in the number of senescent cells in BPH cultures, but not in cancer cultures. Also the hTERT siRNA-treated BPH cells were visibly less confluent than control (Figure 8B). These cells were flatter and contained a large number of vacuoles, other senescence indicators (Campisi, 1997). This suggested that induction of senescence is one of the contributory factors for the reduction in viable cell-count in BPH-derived cultures after telomerase inhibition (**Figure II3.8**).

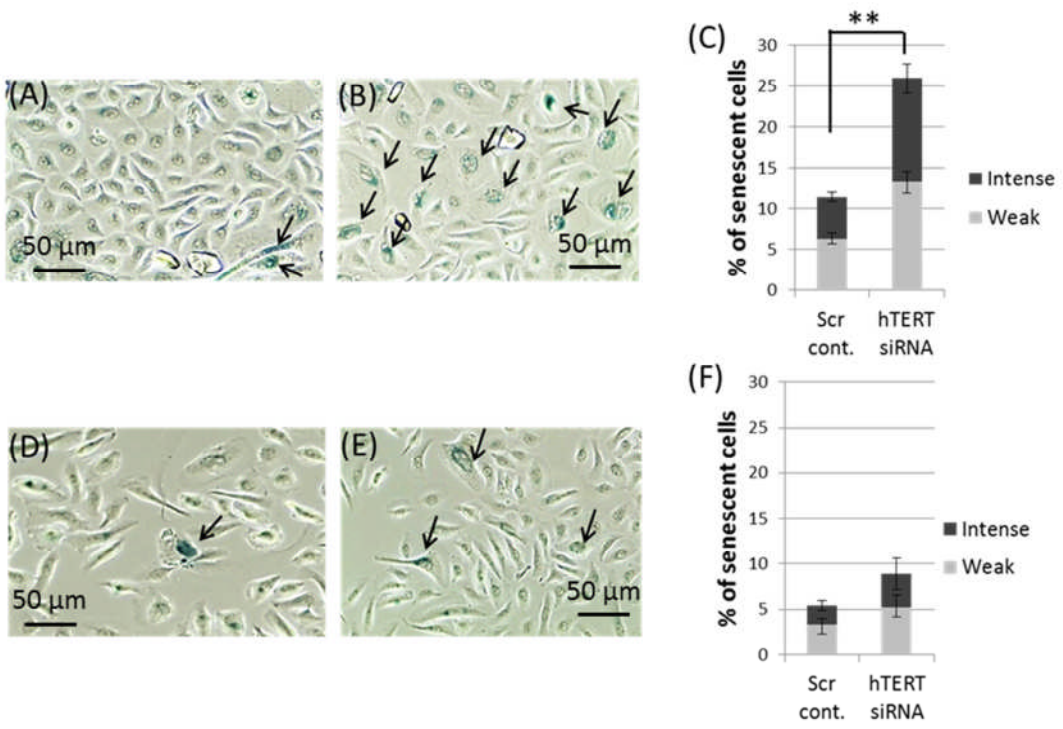


Figure II3.8: Detection of induction of senescence in primary prostate epithelial cultures after telomerase knock-down. Primary BPH (n=5) and cancer (n=3) derived prostate epithelial cells were transfected with scrambled siRNA (Scr) and hTERT siRNA. β-galactosidase staining was performed after 7 days of transfection to detect senescence. Arrows indicate some of the β-galactosidase stained cells. **A**: β-galactosidase staining of BPH derived culture transfected with scrambled siRNA. **B**: β-galactosidase staining of BPH derived culture transfected with hTERT siRNA. **C**: quantification of β-galactosidase stained cells in BPH derived cultures. **D**: β-galactosidase staining of cancer derived culture transfected with scrambled siRNA. **E**: β-galactosidase staining of cancer derived culture transfected with hTERT siRNA. **F**: quantification of β-galactosidase stained cells in cancer-derived cultures. **p<0.01 – paired two-tailed t-test

II3.4.4 Effect of telomerase inhibition on cell proliferation

Another contributory factor towards the reduction of viable cell counts could be a reduction in cell proliferation. To determine the mechanism of telomerase-mediated reduction in cell proliferation after telomerase inhibition for 7 days, Ki-67 immunostaining was used. Ki67 is present only in actively cycling cells (Cattoretti et al., 1992). The positively stained cells were counted manually in 10 20X fields (about 100-125 cells) for quantification (**Figure II3.9**). A slight decrease in number of proliferating cells was observed in BPH culture, whereas a modest increase was noted in cancer cultures after 7-day telomerase inhibition. This difference was not statistically significant. This result suggested that telomerase inhibition for 7 days did not influence cell proliferation in prostate epithelial cultures.

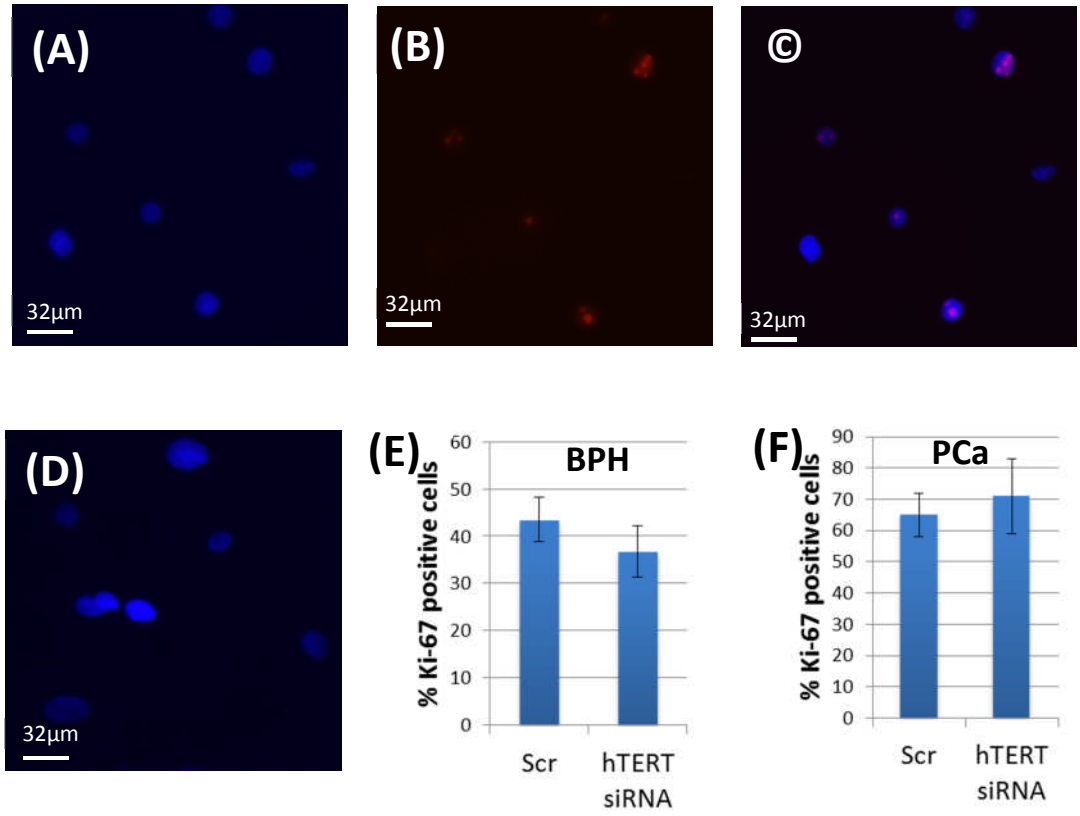


Figure II3.9: Detection of changes in proliferation in primary prostate epithelial cultures after telomerase knock-down. Primary BPH (n=3) and cancer (n=3) derived prostate epithelial cells were transfected with scrambled siRNA (Scr) and hTERT siRNA. Ki-67 staining was performed after 7 days of transfection to label actively cycling cells. Representative images: **A**: DAPI staining. **B**: Ki67 staining. **C**: merge of DAPI and Ki67. **D**: Secondary antibody only control. **E**: Quantification of Ki67 staining in siRNA treated BPH-derived PPECs. **F**: Quantification of Ki67 staining in siRNA treated BPH-derived PPECs. Note that the percentage of Ki67 positive cells are about 20% more in cancer samples compared to BPH samples.

II3.4.5 Effect of telomerase inhibition on colony forming efficiency

Since telomerase was differentially expressed in the SC population enriched from BPH and cancer cultures (**Figure II3.3 and 3.4**), we wished to determine whether its activity was necessary or sufficient for stem cell self-renewal, as indicated by colony forming efficiency. For this purpose, BPH and cancer cultures were transfected with hTERT and control siRNAs. On day 7, 200 cells were plated in 6-well collagen coated plates with irradiated STO's as feeders to assess their colony forming ability. After 2 weeks, colonies were counted under manually light-microscope. Unlike cancer cultures, hTERT siRNA transfected cells derived from BPH cultures formed significantly fewer colonies than that of controls (**Figure II3.10**). Colonies of more than 32 cells indicated more than 5 population doublings, a characteristic of stem cells (Richardson et al., 2004). Colonies with lower number of cells could indicate either a lag in stem cell proliferation or colonies formed by transit-amplifying cells. This result linked telomerase expression to the stem cell self-renewal.

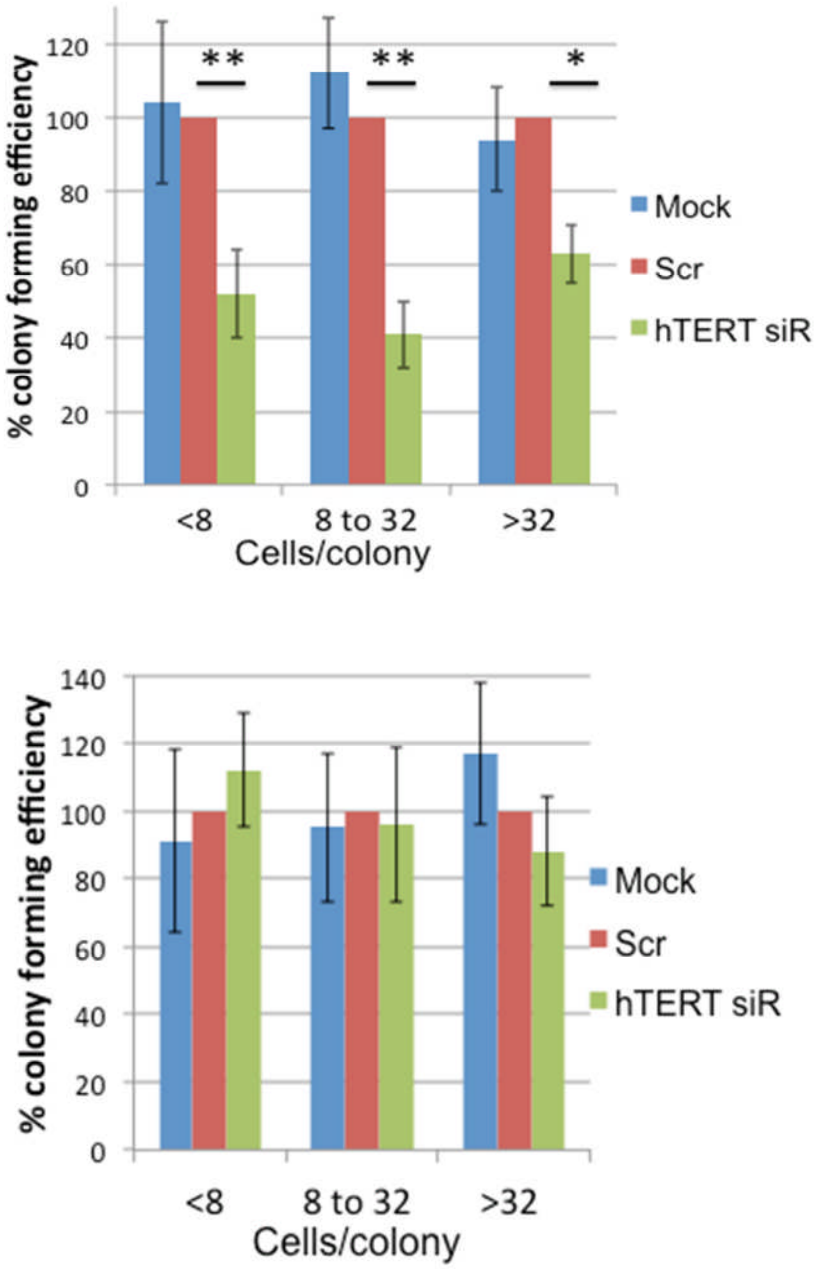


Figure II3.10: Colony forming efficiency of BPH and PCa-derived epithelial cells after hTERT siRNA knock-down for 7 days. Colony forming efficiency of BPH (A, n=4) and cancer (B**,** n=5) cultures after 7-day telomerase inhibition. 200 cells from each of the mock/scrambled/hTERT siRNA transfected cultures were plated in a 6-well plate with irradiated STOs. Colonies were counted on day 14 under light microscope. *p<0.05, **p<0.01 - paired two-tailed t-test

SECTION II: 4. Discussion

In this investigation, we have linked telomerase expression with functional properties of hierarchical subpopulations enriched from normal and pathological primary prostate tissue. The data now sheds light not only the therapeutic usefulness of telomerase in the management of prostate tumours, but also illustrates the contribution of individual hierarchical sub-populations towards prostate tumorigenesis. More specifically, our studies indicate that the BPH is a condition solely sustained by the progenitor compartment hyper-proliferation, and that the acquisition of proliferative potential by differentiated cells is the hallmark of prostate cancer. The stem cell compartment in prostate cancer probably remains quiescent, but can gain telomerase expression/activity when exposed to conditions imparting severe proliferation-driven cellular stress (e.g. CRPCs). Finally, the functional experiments suggest that the non-telomeric role of hTERT could be more predominant in the regulation of telomerase mediated prostate stem cell function. Overall, this analysis, which is performed on pure and homogeneous primary prostate epithelial subpopulations, provides the most definitive and direct assessment of the role of telomerase in prostate tumorigenesis to date.

Telomerase is one of the major players in anti-cancer research owing to its vital location at the crossroads of cancer, stem cell maintenance and differentiation processes (Flores et al., 2006). Therefore, it has been considered as an ideal target for anti-neoplastic therapies. Telomerase is also overexpressed in the vast majority of prostate cancers (80-90%) and was investigated for its therapeutic efficiency in pre-clinical studies (Meeker, 2006). Two recent investigations (published after we started our project) further implied that both prostate cancer stem cells and rest of the tumour bulk cells could be depleted by telomerase inhibition, as the activity was high in the prostate cancer stem cell compartment (Marian et al., 2010, Xu et al.,

2011b). These investigations had some limitations and focussed only on CSCs derived from treatment naïve cancers or cell lines. Therefore, we wanted to explore telomerase expression in all (not just in CSCs) of the prostate epithelial hierarchical subpopulations enriched from normal and pathological (BPH, treatment naïve cancers, and castration resistant cancers) employing patient-derived prostate tissue to obtain a more comprehensive and definitive understanding of the role of telomerase in the prostate epithelial maintenance.

A variety of model systems have been utilised to investigate telomerase in relation to prostate cancer and stem cells. Mouse models have played a vital part in understanding basic telomerase biology and the roles of telomerase in carcinogenesis. But mouse (or rodent) prostate is anatomically, histologically, and functionally very distinct from the human prostate (Shappell et al., 2004). Furthermore mice never develop spontaneous prostate cancers. Differences also exist between human and mouse average telomere lengths and telomerase functionalities. The average telomere length in the majority of established lab mouse strains was found to be considerably longer than wild-derived mice or indeed humans (~30-200kb vs. <20kb) (Zijlmans et al., 1997, Hemann and Greider, 2000, Wright and Shay, 2005). Almost all the TERT knockout mice models also failed to consistently demonstrate the non-telomeric effects of telomerase/TERT at any generation (Blasco et al., 1997, Rudolph et al., 1999, Hao et al., 2005, Majerska et al., 2011, Sahin et al., 2011, Strong et al., 2011). Unlike humans, significant telomerase activity was detected in almost all of the mouse tissues (Prowse and Greider, 1995, Martin-Rivera et al., 1998), and lastly, the DNA damage foci resulting from short dysfunctional telomeres, which increase with age in humans, were not seen in mice (Wright and Shay, 2000, Sedivy, 2007). This suggested that mice might not use telomere shortening as a counting mechanism towards aging. Therefore, in order to comprehend the complete spectrum of human telomerase

functions and draw clinically relevant conclusions, mouse models alone cannot be relied completely. It is absolutely essential to extend functional telomerase investigations to human cell models.

Telomerase activity was also investigated in human prostate cell lines. But this experimental system presented several other challenges. First, the established prostate cell lines have been in culture for years, where they have undergone an intense selection pressure-based on proliferation. As telomerase is one of the most important regulators of cell proliferation, it may be remarkably overexpressed in cell lines and thus may not faithfully represent the original tissue. Telomerase expression in prostate cell lines was indeed found to be significantly higher than in primary tissue (Lin et al., 1997, Soda et al., 2000). Secondly, telomerase expression and telomere length in prostate cancer cell lines have a variable relationship. For example, a comparison between telomerase activity and telomere length in PC3 and LNCaP cells indicated that the cell line with shorter telomeres exhibited the higher telomerase activity (Marian and Shay, 2009, Marian et al., 2010), while, a similar comparison between DU145 and C4-2 cells indicated the exact opposite correlation (Marian et al., 2010). This suggests that the relationship between telomerase activity and telomere length is variable in these cell lines and it is difficult to predict which one better represents that in patient-derived tissue. Lastly, stem cells or TICs isolated from cell lines probably do not represent true cancer stem cells/TICs due to many reasons (as explained in detail in common introduction, page 36). One of them is a lack of tissue homeostasis or niche interaction in cell lines for a prolonged period, which is vital for the identity of stem cells/TICs (Scadden, 2006). For these reasons, we utilised patient-derived primary prostate epithelial cultures (PPECs) for our analysis with occasional comparison with cell lines.

The PPECs were isolated from freshly resected patient tumours by a combination of mechanical and enzymatic separation. These cells were then grown on collagen I-coated dishes for 2 passages. The media used for culture actively promotes the maintenance of basal prostate epithelial cells and removes luminal, endothelial and haematopoietic cells (Collins et al., 2001, Richardson et al., 2004). The cells with stem cell phenotype are extremely rare (~0.1%) in these cultures, so to obtain sufficient number of stem cells (~3000), it was essential to expand cells in culture for 2 passages. But before investigating telomerase in the epithelial sub-populations, we confirmed previous findings in cell lines and made comparisons with unfractionated PPECs.

First, we determined hTERT mRNA levels in several normal, benign, malignant, and hTERT immortalised cell lines. The over-expression of telomerase in prostate cancer cell lines compared to cell lines derived from benign or normal epithelium suggested that telomerase could be important for prostate tumorigenesis. However, the most interesting observation was that not all prostate cancer cell lines had higher telomerase expression than normal cell lines. For example, P4E6 and PC3 cancer cell lines exhibited hTERT expression levels equivalent to that of normal PNT2c2 cell line. The lower expression of hTERT in PC3 compared to LNCaP was also noted in previous investigations (Marian and Shay, 2009). So, as per the commonly held belief, the tumours generated by the cells with low telomerase should be less aggressive and self-confined (Meeker, 2006). But PC3 cells generate by far the most aggressive tumours in immunodefficient mice with higher effectiveness compared to cell lines having relatively higher telomerase expression (e.g. LNCaP cells) (Wu et al., 1994, Mercatelli et al., 2008). These findings emphasise that the higher telomerase expression in prostate cell lines need not necessarily represent higher tumorigenic potential (and vice versa). In addition, androgen dependent cell lines (LNCaP, and VCaP) did not show distinctly different

telomerase expression compared to androgen independent cell lines (PNT2c2, PNT1a, BPH1, P4E6, PC3, and DU145). So in summary, our prostate cell lines analysis showed that hTERT expression cannot be taken as a sole surrogate for tumorigeneic potential and there was no correlation between androgen dependence and telomerase expression.

The analysis of hTERT mRNA levels in PPECs gave a more reliable distinction between different pathological patterns observed in prostate tumours. The PPECs represent mainly the basal compartment of prostate, which is about 50% in BPH and less than 1% in treatment naïve cancer. Even then, the hTERT expression in PPECs faithfully replicated whole tissue analysis findings, ie. hTERT expression in BPH derived PPECs was minimal to absent, but was significantly higher in treatment naïve cancers, and even higher in castration-resistant cancers. The high hTERT expression in CRPC cultures is probably indicative of high proliferation turnover and cellular stress compared to treatment naïve cancers. CRPCs may respond to repeated and prolonged partial ablation of tumours by upregulating hTERT expression to manage added survival challenges. To our knowledge, this is the first investigation assessing telomerase expression in primary CRPC cultures. On further comparisons with cell lines, stark differences between PPECs and the cell lines were evident. PPECs derived from CRPCs exhibited the highest hTERT expression in the primary samples analysis, but this was still lower than hTERT expression in P4E6, the cell line with the lowest hTERT expression in our analysis. This again emphasizes that telomerase is significantly overexpressed in prostate cell lines and may not realistically represent the tissue of origin. Unfortunately, due to limited sample analysis, additional correlations such as with Gleason grade were not possible. Overall, these results confirm the previous expression pattern observed in BPH and cancer samples and also provide additional insights into the telomerase expression in CRPCs. This analysis formed the basis on which we

performed the assessment in the individual hierarchical sub-populations, which identified more subtle features.

We next investigated differential telomerase expression in prostate epithelial hierarchical cell populations. In normal prostate samples, hTERT mRNA levels was undetectable in all sub-populations (SC, TA, and CB), probably depicting the very slow cycling nature of the normal human prostate epithelium. The average division time of an adult rat prostate epithelial cell is about 200 days/cell division (Isaacs and Coffey, 1989) and likely to be slower in human prostate as well. In this scenario, after puberty, normal human prostate epithelial cells, including stem and transit amplifying cells, will undergo fewer cell divisions to maintain the prostate epithelium compared to highly proliferative epithelia like skin or colon. Fewer cell divisions mean a slower telomere attrition rate. It is likely that, at this slower rate of cell division and telomere attrition, normal prostate epithelial cells do not need to activate telomerase, as their telomeres will not reach a critical length. However, this homeostatic arrangement is probably disrupted in BPH, where accelerated cell proliferation exists for a prolonged duration.

The telomerase expression pattern in sub-populations derived from BPH PPECs indicated that BPH could be a disease sustained by the progenitor compartment. In BPH, only the progenitor compartment (SC and TA cells) had elevated hTERT mRNA levels and telomerase activity. In benign lesions, the cells that naturally possess high proliferative potential (SC ad TA cells) (Richardson et al., 2004) proliferate for extended time (years) and maybe at a slightly higher than usual pace. Owing to this prolonged and high proliferation rate, these cells may erode their telomeres, which can then activate telomerase expression. If this hypothesis is correct, then SC and TA populations from BPH should have shorter telomeres. Alternatively, telomerase and hTERT expression have been shown to be necessary for progenitor cell maintenance in multiple systems (Hiyama and Hiyama, 2007,

Choudhary et al., 2012). The rapid increase in progenitor cell pool in BPH may require hTERT and telomerase expression at the very beginning of the pathology. High telomerase activity can then prevent proliferation dependent telomere shortening. In this scenario, the telomeres in progenitor cells could be near to normal length. Unfortunately at this moment, there is no information about the telomere length in BPH derived epithelial sub-populations to support any of the above hypotheses. Determination of telomere length in BPH-derived epithelial sub-populations in future analysis would provide a substantial supportive evidence for this hypothesis. Based on this hypothesis, it could be assumed that the progenitor compartment is expanded in BPH. These cells then give rise to more differentiated cells and the differentiated cells per se, do not exhibit any pathological proliferation. The acquisition of hTERT expression and proliferative capacity by terminally differentiated luminal cells is perhaps indicative of cancer development.

Indeed, the differentiated cell sub-populations (CB and LC) enriched from primary treatment naïve cancer samples exhibited high telomerase expression and activity. In normal tissues, these cells have minimal, or no proliferative potential, but in cancer, these cells are highly proliferative (Tu et al., 1996). The gain of hTERT expression in differentiated cells seems to be a distinctive feature of cancer, where cells that are not programmed to proliferate acquire an abnormal proliferative potential. The cancer TA sub-population also exhibits telomerase expression and activity. So in cancer, all the sub-populations except stem cells have active telomerase and presumably can proliferate significantly. In such a scenario, more than 99% of cancer cells possess abnormal and independent proliferative potential. Therefore, stem cells do not remain under any obligation to provide a cellular pool for the maintenance of the expanding cancer mass. The increasing number of differentiated cells may also send a negative feedback to stem cells to inhibit their proliferation. This may force cancer stem cells into quiescence where no telomerase

expression or activity is required. In fact, the quiescent nature of cancer stem cells is proposed as one of the important contributory factors for their drug resistance (LaBarge, 2010, Li and Bhatia, 2011, Borst, 2012).

Our results are at odds with several previously published findings, where the investigators suggested that telomerase activity in prostate cancer stem cells is equivalent (Marian et al., 2010) or significantly higher (Xu et al., 2011b) than non-CSC populations. Marian et al. demonstrated that telomerase inhibition by imetelstat can target prostate TICs and non-TICs effectively (Marian et al., 2010). They utilised the established prostate cancer cell lines (DU145, C4-2, and LNCaP) to isolate prostate TICs based on cell surface marker expression (CD44, integrin $\alpha_2\beta_1$, and CD133 – the same markers as used in our analysis), Hoechst 33342 dye exclusion, and holoclone formation assay. Telomerase activity was found to be 10-15% lower in TICs vs. non-TICs and in holoclones (colonies generated by TIC-like cells) generated from DU145 cells compared to colonies generated from whole population. The authors showed that survival of both of these populations could be significantly inhibited (~90% reduction) by treatment with telomerase inhibitor imetelstat for 72 hrs. Interestingly, the authors found that the telomere length in TICs and non-TICs was similar but the telomere length in holoclones was shorter than that of whole population. This discrepancy was not discussed in the paper. Additionally, the authors showed that all the TICs exhibited a high telomerase activity, but they could only abolish 50% of the TICs even after prolonged telomerase inhibition (for 110 days). Again, the possible mechanisms behind this result were not explained. Based on these results, the authors conclude that: “In summary, this preclinical study shows that telomerase inhibition has a great potential for the treatment of prostate cancer and may be able to target the TICs that contribute to relapse and metastasis (Marian et al., 2010).” This conclusion was solely based on the data generated using cell line models, and the publication had

few unexplained questions (as discussed above). Therefore, this investigation may not accurately represent telomerase biology in primary patient prostate cancer.

On the other hand, the study from Xu et al., analysed primary human prostate cancer cells for telomerase expression in TICs. This data leads to very different conclusions (Xu et al., 2011b). In their analysis, they provided evidence for high telomerase expression and activity specifically in TICs, using patient derived tissue material and an in vivo mouse model (Xu et al., 2011b). This is in contrast with the findings from Marian et al. where they showed that progenitor cells (TICs) have 15-20% *less* telomerase activity than the rest of the tumour cells. The authors suggest that telomerase is specifically overexpressed in cancer progenitor cells and it can be successfully targeted to significantly inhibit tumour growth in vivo. Although interesting, this study leaves some unanswered or unexplained questions. The concerns about this study are: (i) cells were isolated directly from tumours, without culturing. In our experience, this method limits the purity of cells as some luminal and inflammatory cells invariably piggyback to progenitor cells. (ii) The authors used 1 hr collagen adhesion, whereas, the original investigators recommend 5-20 min of adhesion for $\alpha_2\beta_1^{hi}$ selection. This again limits the purity of progenitor cells and was evident in their experiments, where more than half of their progenitor cells failed to form colonies or demonstrate invasion. (iii) Xu et al. proposed that only progenitor cells (which formed 0.7–9.2% of the total mass in their samples) expressed telomerase. This did not explain the widespread hTERT tumour immunoreactivity demonstrated in previous study (Iczkowski et al., 2002). Also, the telomerase expression in fewer than 10 % of cells failed to explain how this restricted expression can impart strong positivity to the entire cancer sample. The lower expression of telomerase in the non-progenitor population also did not match with the previous investigation (Marian et al., 2010). (iv) The authors could not explain the similarity between telomere length observed in progenitor and non-progenitor

cells even when they displayed a remarkable difference in telomerase expression. (v) It seems unlikely that mutant telomerase can induce apoptosis in 4 days through telomere attrition, suggesting that this action may be mediated by non-telomeric function of hTERT. This possibility was not discussed and therefore, remains unexplored. (vi) The mutant hTERT transfected xenografts showed an extraordinary long lag period (about 66 days compared to 35 days in control arm), suggesting that the mutant hTERT may have also inhibited stem cell self-renewal. This possibility also remains unexplored in both models.

One or more of the above-mentioned concerns, also hampered investigations in other organs that showed higher telomerase activity in CSCs. At least four independent studies involving brain, pancreas, breast, and lung cancer proposed that telomerase is over-expressed CSC population and that these cells can be depleted by inhibition of telomerase (Joseph et al., 2010, Beck et al., 2011, Castelo-Branco et al., 2011, Serrano et al., 2011). All of these investigations were based upon CSCs enriched from cell lines, and the results were not validated in patient-derived tissue material. The contrasting results of telomerase expression in neuronal CSCs also emphasised the inability of cell line models to deliver consistent and comparable results in this field (Shervington et al., 2009, Castelo-Branco et al., 2011). Due to the limitations of cell lines in investigations of telomerase biology (as discussed before), the above-mentioned studies need further validation in patient-derived tissue material and/or in animal models. Therefore, in contrast to these investigations, we have based our analysis on tissue material from patients and supplemented it with human tissue-derived mouse xenograft analysis.

Our analysis further showed that telomerase is significantly over-expressed when cells have to proliferate under alien or stressful conditions e.g. in xenografts and CRPC (environment probably similar to cell lines!). hTERT expression was significantly up-regulated in all the sub-populations derived from CRPC PPECs and

xenografts. Androgen ablation therapy continuously and repeatedly ablates androgen expressing differentiated luminal cells, in a process that inevitably leads to CRPC. This probably sends feedback signals to trigger proliferation of quiescent cancer stem cells. An expansion of the stem cell compartment has indeed been noted in the skin epithelium after wounding (Blanpain and Fuchs, 2009, Lu et al., 2012) and in multiple cancers after conventional chemotherapeutic treatments (Dylla et al., 2008, Ma et al., 2008, Gupta et al., 2009, Hoey et al., 2009, Mueller et al., 2009, Calcagno et al., 2010). Even in prostate cancer, an increase in the basal cell content (that harbours stem cells) was observed after androgen ablation (Gil-Diez de Medina et al., 1998, van Leenders et al., 2001a, Maitland et al., 2011). So in CRPCs, the rapidly proliferating, telomerase positive and AR negative populations (SC, TA, and CB) perhaps attempt to compensate for the continual loss of differentiated cells. When prostate cells are engrafted subcutaneously into immunocompromised mice, the progenitor compartment is probably similarly forced to proliferate. Owing to their advantageous traits, such as significant proliferative potential, self-renewal ability, niche adaptability, and defences against DNA damaging agents; the progenitor cells are probably better equipped to adapt to an alien environment (Wennersten et al., 2006, Dylla et al., 2008). The commonest phenotype observed in xenografts generated in our lab is indeed composed of undifferentiated cells (Maitland et al., 2011). The severe proliferation dependent selection pressure forces all the sub-populations enriched from CRPCs and xenografts, including stem cells, to proliferate at a higher pace. The differentiated cells may not cope with the interventions or the surrounding foreign environment, leading to aberrant differentiation and relative expansion of the progenitor compartment. These results signify that the microenvironment of cells can significantly influence telomerase expression.

The functional experiments conducted on the BPH and PCa-derived PPECs showed that telomerase inhibition for 7 days could significantly inhibit unfractionated BPH PPEC growth and colony forming efficiency, and induce senescence irrespective of lower telomerase expression and activity in them (compared to PCa-derived PPECs). Telomere attrition usually requires telomerase inhibition for weeks to months (Marian et al., 2010, Roth et al., 2010), whereas non-telomere related effects of hTERT are likely to be apparent within a week of hTERT inhibition. We did not perform a correlation with telomere length in our studies, but because the above mentioned effects in BPH-derived PPECs were observed within a week of telomerase inhibition, it is very likely that these effects could primarily be mediated through non-telomeric functions of the hTERT subunit. A comparison of telomerase expression/activity data with these functional studies suggested that the observed functional changes could be mediated through interference in hTERT in prostate epithelial progenitor cells.

The expression/activity analysis has demonstrated that telomerase is expressed only in the progenitor compartment (SC and TA cells) of BPH. These cells constitute less than 5% of the cells the tissue and about 20-30% of PPECs, and are principally responsible for the growth of BPH PPECs in a monolayer (Collins et al., 2001, Richardson et al., 2004). On inhibition of telomerase, we predicted that the proliferative potential of these progenitor cells was likely to be reduced. In fact, a 40% reduction in the number of viable cells was observed after 7-days of telomerase inhibition. This reduction could at least partly be due to the induction of senescence and partly due to reduction in their colony forming efficiency. There was also a small decrease in the proliferating cells, but the reduction was not statistically significant. These findings indicate that telomerase, and more specifically hTERT, is necessary for BPH progenitor cell proliferation and self-renewal. During a week, the BPH PPECs undergo 2-4 cell divisions (approximate personal observation) and

therefore, the absence of telomerase for 7 days is unlikely to reduce telomeres to the critical length to cause telomere-mediated senescence. So, we propose that the observed effects of telomerase inhibition in BPH PPECs are mediated through non-telomeric functions of hTERT. The inhibition of hTERT may interfere with mitochondrial free radical scavenging pathway, increasing the levels of intra-cellular free radicals (Sahin et al., 2011). This continued oxidative insult may then result induction of senescence (Cristofalo et al., 2004). In addition, disruption of hTERT interactions with several signalling pathways (e.g. WNT and TGF-β) is capable of reducing stem cell self-renewal capacity and proliferation (Cong and Shay, 2008). The telomere independent effects of hTERT inhibition on stem cells and mitochondria have indeed been well studied in multitude of systems (Park et al., 2009, Sahin et al., 2011, Sharma et al., 2012). Therefore, although these lines of evidence need to be confirmed by a direct demonstration of telomere-independent hTERT association with the key pathways involved, it seems more than plausible that non-telomeric functions of hTERT are essential for BPH derived SC maintenance.

On the other hand, treatment naïve cancer derived PPECs were not affected by telomerase inhibition. The viable cell count decreased marginally, and there was minimal induction of senescence. The reduction in cell proliferation and colony forming efficiency was not observed as well. There could be two explanations for these observations. First, the number of cells expressing telomerase in cancer, together with the overall telomerase activity, is much higher than in BPH. In a situation where we used similar concentration of siRNA for equal number of BPH and cancer PPECs, the effect of the siRNA could be diluted in cancer PPECs. The percentage reduction in telomerase levels, in cancer PPEC was similar to that observed BPH PPEC. However, due to the initial higher expression in cancer PPECs, the residual telomerase expression after knockdown was still quantitatively

higher. This relatively high telomerase expression after siRNA transfection may have stifled the effects of hTERT inhibition in cancer PPECs. Second, treatment naïve cancer stem cells do not exhibit telomerase expression. So telomerase inhibition would not affect these cells. The inhibition of telomerase in the rest of the cell populations may result in rebound stem cell division, giving rise to more telomerase negative proliferating cells. These cells may compensate for the dying (not senescent) differentiated cells. It should be noted that there is 1.1-1.15 fold increase in the proportion of proliferating cells in cancer PPECs after telomerase inhibition. This increase could be partly due to the relative increase in rebound proliferating stem cells, but more focussed experiments are necessary in order to dissect this response. These results, along with lack of telomerase expression/activity in CSC population enriched from PCa, suggested that telomerase inhibition would not be an efficient treatment in treating treatment naïve prostate cancers. These results also indicate that telomerase expression is not essential for prostate CSC survival. Hence, although CSCs enriched from CRPCs express high telomerase, they may not be effectively eliminated by telomerase inhibition.

To conclude, telomerase inhibition for 7 days has contrasting effects on BPH and treatment naïve cancer PPECs. Considering that BPH stem cells express telomerase and cancer stem cells do not, the failure of telomerase inhibition to influence cancer PPECs suggest that the effects seen in BPH could be mainly due to the impact on BPH stem cells (**Figure II4.1**). The hTERT subunit can interact and influence pathways such as WNT, TGF-β, Notch, c-MYC, and STAT3 that can regulate stem cell fate (Flores et al., 2006, Jones and Wagers, 2008). We hypothesize that inhibition of these non-telomeric interactions underpin the effects seen in BPH PPECs. These results also suggest that telomerase inhibition, as a stand-alone therapeutic tool, may not work in cancer, as cancer stem cells won't be

targeted. Telomerase inhibition could represent a valid strategy in patients with CRPCs, but it is important to confirm the *dependence* of CRPC CSCs on telomerase. However, all these propositions need further investigations to obtain direct evidence for the underlying mechanisms.

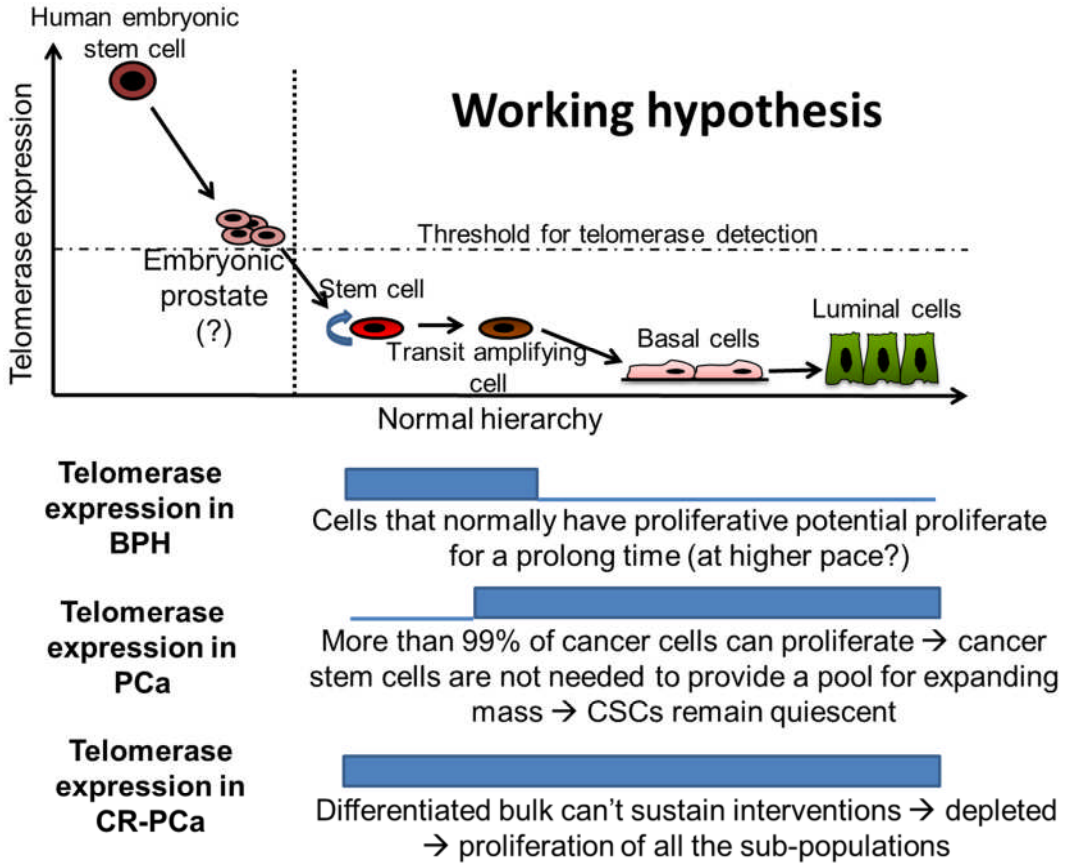


Figure II4.1: Working hypothesis explaining telomerase expression/activity and its possible impact on cellular dynamics in prostate pathologies.

Future work:

It would be interesting to identify the precise mechanism of action of hTERT-mediated regulation of self-renewal of stem cells enriched from BPH PPECs. For that purpose, correlation with telomere length and with non-telomeric hTERT interacting pathways (especially TGF-β and WNT signaling pathways) would provide additional insights. In order to assess the impact of telomerase inhibition on

telomere length, long-term telomerase inhibition by a proven specific and potent small molecular inhibitor, such as imetelstat, would probably be more suitable than multiple siRNA or even shRNA transfection (We have spoken with Prof. Jerry Shay, who has kindly agreed to help us in this regard). The identification of precise effect of telomerase inhibition on individual epithelial sub-population enriched from BPH, PCa, and CPRC in relation with stromal co-cultural (to account for stromal influence on telomerase signalling) would certainly increase our understanding of telomerase biology in prostate pathologies.

Section III: miRNA profiling of prostate epithelial sub-populations

SECTION III: 1. Introduction

Two key research priorities in aggressive prostate cancer management are the identification of aggressive cancers at diagnosis and the design of efficient and individualised treatment plans. Novel therapeutics like Abiraterone acetate and MDV3100 and prognostic indicators (changes in circulating tumour cell count, PSA progression free interval etc.) are beneficial in a sub-set of patients (de Bono et al., 2011, Armstrong et al., 2012, Scher et al., 2012b); however, it has not been possible to identify these sub-sets at or before the start of the treatment. In this scenario, the emerging role of microRNAs (miRNAs) in therapeutics and prognosis make them prime candidates for investigation. miRNAs are well conserved, endogenously encoded, single-stranded small (21-23 nucleotides) non-coding RNAs (Bartel, 2004). They play essential roles in development and disease by regulating gene expression (Ambros, 2004). miRNAs are also secreted in body fluids, are stable, and have a tissue-specific expression patterns (Soifer et al., 2007). These properties of miRNAs can be readily exploited to develop targeted therapies with minimal side effects and relatively easily detectable biomarkers for disease progression and treatment response.

During the last two decades, miRNAs have attained prominence owing to their critical role in post-transcriptional gene regulation. Ambros and colleagues were the first to discover that the LIN-4 gene product, which regulates lineage specification through lin-14, did not code for a protein in *C. elegans* (Lee et al., 1993). Instead, lin-4 encodes for 61 nucleotide (nt.) long precursor RNA with a stem loop structure and a smaller ~22 nt. long functional RNA. The smaller lin-4 RNA product had anti-sense complementarity for multiple sites in the 3' untranslated region (UTR) of lin-14 mRNA and could reduce lin-14 protein expression without reducing lin-14 mRNA levels (Lee et al., 1993, Wightman et al., 1993). These initial studies conclusively

established that lin-4 could regulate gene expression by antisense RNA-RNA interaction, also known as RNA interference (RNAi). The ~22 nt. long lin-4 RNA is now considered the founding member of the miRNA family of small non-coding RNAs.

III1.1 miRNA biogenesis, maturation and degradation

The transcription of miRNAs from miRNA genes is only slightly different from classical protein-coding mRNA transcription and is conserved in the animal kingdom with minimal differences. There are about 1600 annotated human miRNAs (miRBase, 2012). The location of miRNA coding genes is variable, ranging from intergenic region (in introns or exons), antisense orientation to neighbouring genes, or as a solo miRNA gene (Cai et al., 2004, Lee et al., 2004, Rodriguez et al., 2004, Kim and Kim, 2007). Several miRNAs can also be synthesised from the same miRNA gene. miRNA genes may have (i) their own promoters, (ii) a common promoter for clustered miRNAs, or (iii) they can be transcribed along with the surrounding host gene (Kim and Kim, 2007, Ozsolak et al., 2008, Berezikov, 2011). The origin of miRNAs as a part of polycistronic cluster does not necessarily mean that the mature miRNAs of a cluster will be homologous in structure and function (Altuvia et al., 2005). All these miRNAs are transcribed as double-stranded stem-loop precursor miRNA (pri-miRNA) with 5' cap and 3' poly-A tail by RNA polymerase II (Cai et al., 2004, Lee et al., 2004, Zhou et al., 2007). The pri-miRNAs then undergo a series of post-transcriptional modifications to form the mature single stranded miRNAs.

The processing of pri-miRNA is a three-step process (**Figure III1.1**). First, a member of the RNase III family of enzymes, Drosha, cleaves the pri-miRNA at the base of stem loop (Denli et al., 2004). Drosha exists as a part of the microprocessor complex, which identifies its substrate (pri-miRNA) owing to its tertiary structure

(Lee et al., 2003, Zeng et al., 2005). The cleavage product of this complex is a ~70-nt. precursor miRNA (pre-miRNA) (Lee et al., 2003, Denli et al., 2004). The 5' and 3' remnants of the cleavage process are thought to be degraded in the nucleus. The clustered miRNAs that are transcribed together as a common pri-miRNA also get cleaved in the nucleus into individual pre-miRNAs, although, the exact mechanism for this is yet to be explored. Some miRNAs that are embedded within the short introns of protein coding mRNAs, mirtrons, bypass the Droasha cleavage (Okamura et al., 2007, Ruby et al., 2007). The pre-miRNAs for these mirtrons are formed during the splicing of the concerned mRNA introns. Once the pre-miRNA is formed through any of these methods, it is then transported to the cytoplasm through an interaction with Ran-GTP and exportin-5, (Bohnsack et al., 2004).

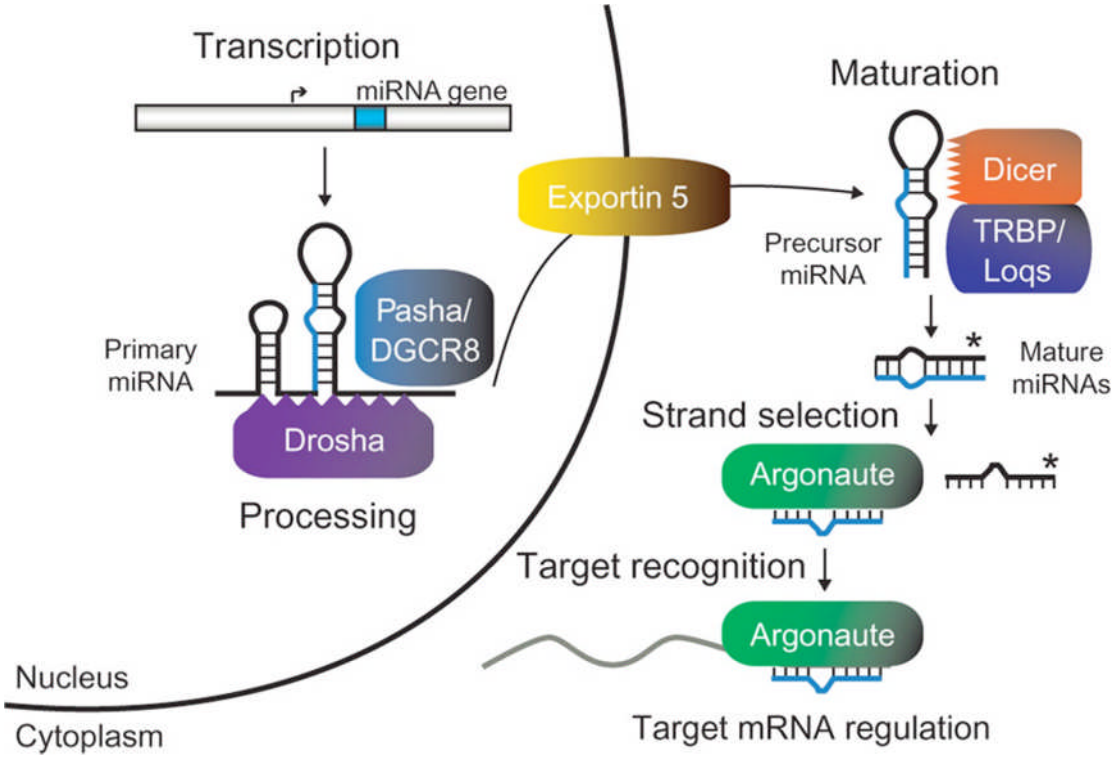


Figure III1.1: miRNA biogenesis and maturation. miRNA is transcribed as primary miRNA (pri-miRNA), which is first cleaved into precursor miRNA (pre-miRNA) in the nucleus by a microprocessor complex containing Drosha. After nuclear export, Dicer containing complex cleaves pre-miRNA into 2 mature single stranded miRNAs. One of the miRNA strands gets incorporated in Argonaute containing RISC complex to regulate mRNA levels (Kai and Pasquinelli, 2010).

In a second step, a multi-protein pre-miRNA processing complex containing Dicer cleaves the pre-miRNA into two complementary single stranded mature miRNA strands (Bernstein et al., 2001, Hutvagner et al., 2001, Knight and Bass, 2001). Although alternative pathways exist for pre-miRNA cleavage, Dicer incises the vast majority of the pre-miRNAs near the terminal loop to form transcripts of about 22 nt. length (Cheloufi et al., 2010, Cifuentes et al., 2010). One of these strands with relatively higher thermodynamic stability was considered as a dominant guide miRNA strand, whereas the remaining strand was considered redundant (Kim et al., 2009). The redundant passenger strand was denoted by the * sign; for example, the redundant strand for miR-1 is miR-1*. However, recent evidence showed that in some cases, the passenger strands (miR* sequences) also have a functional role (Czech and Hannon, 2011, Yang et al., 2011). The miRNA nomenclature has been changed to adapt to these findings, whereby miRNA strands are now referred to as miR-x-3p and miR-x-5p, based on their orientation (miRBase, 2011). Both nomenclatures are currently in use.

The third and the final step in the miRNA processing is the incorporation of the mature single stranded miRNA strand into the RNA-induced silencing complex (RISC). The multi-protein pre-miRNA processing complex forms a vital component of the RISC loading complex. One of the mature miRNA strands (the guide strand), selected on its thermodynamic stability and position within the stem loop, is incorporated in the RISC assembly (Khvorova et al., 2003, Schwarz et al., 2003, Krol et al., 2004, Lin et al., 2005). The RISC loading assembly incorporates the preferred miRNA strand on the core RISC effector protein, Argonaute-2 (Ago2) (Liu et al., 2004, Meister et al., 2004). RISC with the incorporated miRNA is sometimes referred to as the miRISC assembly, which determines the fate of target mRNAs. The abundance and stability of miRNAs depend upon transcriptional regulation,

post-transcriptional modifications, such as RNA editing, and numerous feedback loops involving miRNA and their target mRNAs (Kim et al., 2009).

III1.2 Mechanism of action of miRNAs

The miRISC complex interacts with specific target mRNAs to prevent their translation. The identification of target mRNAs is largely determined by a partial miRNA sequence homology with the 3' UTR of the mRNAs (Bartel, 2009). A perfect complementarity between the full miRNA sequence and the target mRNA 3'-UTR (for example, between miR-196 and HOXB8 mRNA) is seen in extremely rare cases in animals (Yekta et al., 2004). However, the vast majority of miRNA-mRNA interactions are determined by perfect binding between 3' UTR of mRNA and nucleotides 2 to 7 at the 5′ end of the miRNA, called the 'seed' region (Lewis et al., 2005). Some experiments show that the seed pairing is necessary and sufficient for miRNA-mediated mRNA regulation (Doench and Sharp, 2004). Multiple bioinformatic prediction algorithms, such as TargetScan, miRanda etc., can now identify prospective mRNA targets based upon seed sequence directed miRNA-mRNA interactions (Bartel, 2009). Recent investigations demonstrate even more flexibility in miRNA-mRNA interactions, where extensive binding outside the seed region can compensate for imperfect seed sequence pairing (Reinhart et al., 2000, Slack et al., 2000, Shin et al., 2010). Other studies even suggested that miRNAs could regulate mRNA fate by binding outside the 3' UTR (Tay et al., 2008). Flexibility created by these mechanisms results in the existence of thousands of potential mRNA targets for one miRNA and a possibility for a single mRNA to be targeted by multiple miRNAs.

The precise mechanism used by mammalian miRNAs to regulate target mRNAs are still hotly debated, as there is evidence for multiple mechanisms (Huntzinger and Izaurralde, 2011):

- Inhibition of translational initiation
- Inhibition of translational progression
- Deadenylation and degradation
- Endonucleolytic cleavage
- Stimulation of translation/transcription

A rare perfect pairing of a miRNA with its target site on the mRNA results in Argonaute-mediated mRNA endonucleolytic cleavage (Llave et al., 2002, Yekta et al., 2004); while degradation of the target mRNA is probably the commonest mechanism for miRNA-mediated gene silencing (Huntzinger and Izaurralde, 2011). Specific miRNA-target association studies and general transcriptome analysis demonstrating target mRNA downregulation after miRNA transfection, suggested that miRNAs could induce targeted mRNA degradation (Bagga et al., 2005, Selbach et al., 2008, Hendrickson et al., 2009). In these cases, miRNAs direct their target mRNAs to 5'-to-3' mRNA decay pathway, where mRNAs are degraded after removal of the poly(A) tail and decapping (Rehwinkel et al., 2005, Behm-Ansmant et al., 2006). miRNA-mediated gene silencing can also be achieved by repression of mRNA translation before or after translational initiation and mRNA destabilisation (Nottrott et al., 2006, Mathonnet et al., 2007). Some isolated reports also indicate that miRNAs can promote gene expression by inducing translation (Vasudevan and Steitz, 2007) and by direct interaction with the target mRNA promoter (Place et al., 2008). In summary, miRNAs mainly repress gene expression to influence a wide variety of cellular events.

III1.3 miRNAs in development and disease

miRNAs are proposed to regulate expression of more than 60% of the proteins and thereby, can influence almost every cellular process in mammals (Friedman et al., 2009). They preferentially target genes involved in signalling processes over genes

with housekeeping functions, and orchestrate temporal developmental programs by regulating genes involved in particular biological functions (Zhang et al., 2009). It was proposed a few years ago that miRNAs can either switch targets off or fine tune their expression (Bartel and Chen, 2004). Switching off targets could change cell fate, whereas fine-tuning target expression may smooth out fluctuations in their expression and can also direct dose-dependent effects (Mansfield et al., 2004). Fine-tuning target mRNA levels appears to be the commonest mode of miRNA function, as mutations in relatively few miRNA genes produced robust abnormal phenotypes in *C. elegans* (Miska et al., 2007). Using these two mechanisms, miRNAs can direct several vital developmental activities such as: (i) embryonic survival, (ii) patterning and morphogenesis, (iii) defining and maintaining lineage specification, and (iv) proliferation and apoptosis (Bushati and Cohen, 2007). Often, miRNAs are also involved in a feedback/feedforward loop with their targets, and transcriptional regulators of miRNA expression to ensure precision in the regulation of these vital events (Tsang et al., 2007, Botchkareva, 2012). Imbalance in these delicate arrangements frequently results in developmental defects and diseases.

III1.4 miRNA alterations in prostate cancer

miRNAs are an intense area of research in the prostate. However, the focus is mostly on prostate cancer, and there is no or minimal information about the role of miRNAs in the normal human prostate development and homeostasis.

Alterations in miRNA copy number and changes in miRNA epigenetic regulation are observed in almost all cancers, including prostate cancer (Zhang et al., 2006, Ryan et al., 2010). The up-regulation of the miRNA processing enzyme Dicer in a stage and Gleason-grade dependent manner in prostate cancer possibly imply that gradual and considerable global miRNA expression changes could take place during prostate carcinogenesis (Chiosea et al., 2006). Several studies in the last 5-7

years produced a wealth of data that highlight a critical role for specific miRNAs in prostate cancer initiation, progression and relapse (**Figure III1.2**). These investigations ascertained the oncogenic and tumour-suppressor nature of certain miRNAs and also pinpointed the potential of miRNAs as personalised therapeutic targets and diagnostic/prognostic indicators.

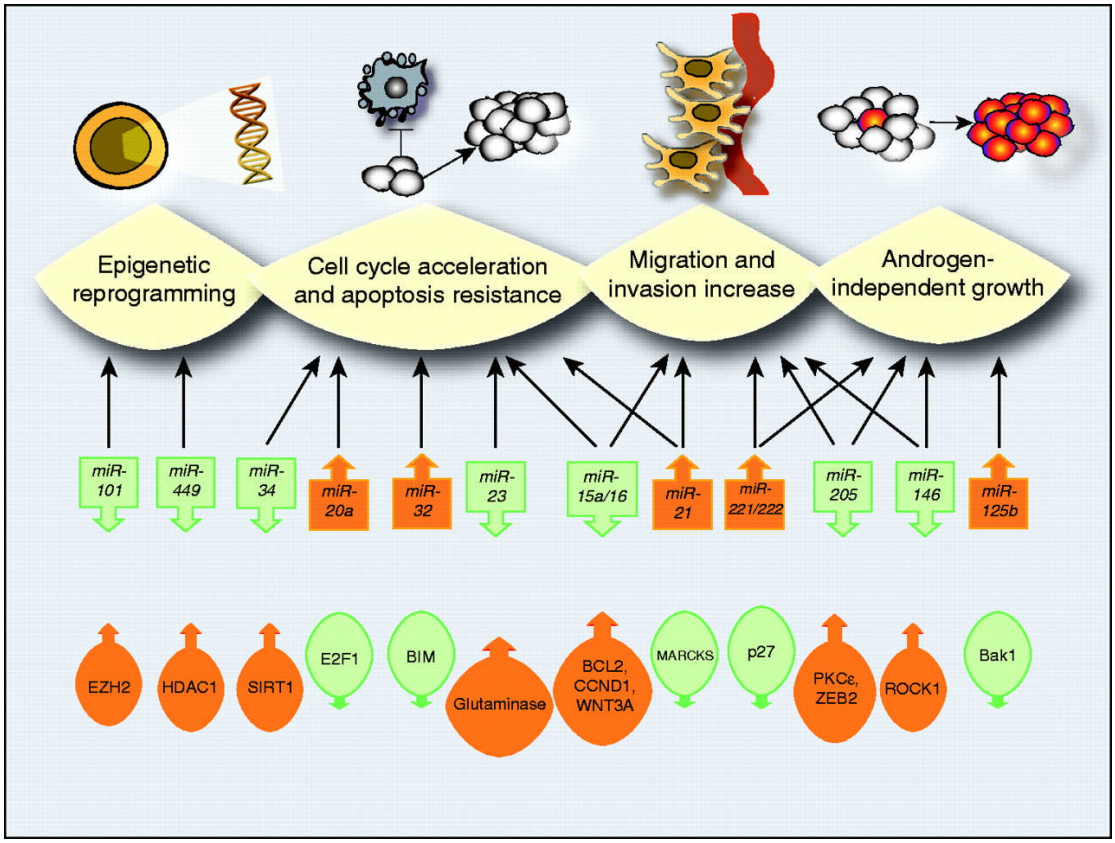


Figure III1.2: The role of miRNAs in prostate cancer. miRNAs can alter any of the processes involved in carcinogenesis to positively on negatively impact on tumour initiation, progression and metastasis. In this diagram, miRNAs are indicated by squares and their mRNA targets by ovals. Green colour indicates a tumour-suppressor nature and orange colour indicates an oncogenic function. The direction of the arrows indicates expression status in cancer compared to the normal prostate tissue. Adapted from (Coppola et al., 2010).

Two principal approaches were taken to identify the functions of miRNAs in prostate cancer: (i) candidate miRNA-specific detailed functional studies and (ii) genome wide assessment using miRNA microarrays. Investigation of a specific miRNA can identify its functional effects in detail, but the scope of such investigations remains

seriously restricted and may be biased towards 'favourite' miRNA analysis. Microarray studies, on the other hand, can provide unbiased genome-wide information about the alteration in miRNA machinery in cells. However, transcriptome-wide miRNA microarray studies in prostate cancer are not precisely informative, as there is a wide disagreement between miRNA microarray data generated by different laboratories (Porkka et al., 2007, Ambs et al., 2008, Ozen et al., 2008, Tong et al., 2009, Schaefer et al., 2010a, Szczyrba et al., 2010, Catto et al., 2011, Martens-Uzunova et al., 2012). These variations could be due to differences in study design, underestimated treatments of patients, methods of sample collection, degrees of tissue heterogeneity, and the differential sensitivity and specificity of microarray platforms used (Coppola et al., 2010). As miRNAs have distinct cell-type specific expression patterns, the differences in differentiation states of tumours, epithelium/stroma ratio, and presence of contaminating cells such as normal prostate epithelial cells, fibroblasts, endothelial cells, and inflammatory exudates may have seriously affected the microarray profiles in these investigations. In spite of these differences, there is a relative consensus about the role of certain miRNAs in prostate carcinogenesis. These miRNAs are discussed in next sections.

III1.4.1 Oncogenic miRNAs (oncomiRs) in prostate cancer

miRNAs, such as miR-221, miR-222, miR-21, and miR-125b, which can inhibit apoptosis or promote cell multiplication, are upregulated in prostate cancer (Catto et al., 2011). These miRNAs can also promote prostate cancer metastasis (Catto et al., 2011). The common feature of most of the oncogenic miRNAs in prostate is that they are proposed to be regulated by androgens, but at the same time, are up-regulated in androgen refractory setting (as described below). Another recurring feature is that none of these miRNAs could be definitively labelled as oncogenic miRNAs in prostate cancer, as contradictory data almost always exists (Coppola et

al., 2010). This suggest that these miRNAs have yet unidentified regulators and downstream targets.

miR-221 and miR-222 are often considered together, due to their sequence homology and overlapping functions. These two miRNAs are over-expressed in multiple cancers, such as glioblastoma, melanoma, and cancers of liver, thyroid, kidney and bladder, stomach, and pancreas (Coppola et al., 2010). The up-regulation of miR-221 and miR-222 was also noted in treatment naïve prostate cancer (Galardi et al., 2007, Mercatelli et al., 2008, Sun et al., 2009) and in castration resistant xenografts and CRPCs (Sun et al., 2012) compared to BPH samples. They potentially promote prostate epithelial proliferation through inhibition of $P27^{Kip1}$, $P57^{Kip2}$, and ARHI (Galardi et al., 2007, Sun et al., 2009, Chen et al., 2011) and cell migration and invasion through inhibition of DVL2, a WNT signalling mediator in prostate cancer cell line models (Zheng et al., 2012). It was also shown that androgens could directly suppress the expression of miR-221 and miR-222 (Ambs et al., 2008). Treatment naïve prostate cancer almost always has activated androgen signalling (Dehm and Tindall, 2005). In this scenario, concurrent overexpression of androgens and miR-221/-222 is puzzling. Further confusion was created by studies showing downregulation of these miRNAs in a subset of prostate cancers, including TMPRSS2:ERG fusion positive cancers (Gordanpour et al., 2011, Jalava et al., 2012). Detailed functional evaluation in patient-derived tissue and in in-vivo models may assist in identifying the exact role and mRNA mediators of these miRNAs in the development and progression of prostate cancer.

Another oncogenic miRNA in prostate cancer is miR-21. This miRNA is more consistently up-regulated in prostate cancer (Coppola et al., 2010). The principal processes promoted by miR-21 overexpression are apoptosis resistance and cell migration (Hassan et al., 2012). It can influence the expression of tumour suppressors such as TPM1, PDCD4, MARCKS, SPRY1, and RECK in prostate

cancer cell lines; consequently inhibiting apoptosis and promoting growth in vitro and in vivo (Li et al., 2009, Darimipourain et al., 2011, Li et al., 2012, Reis et al., 2012). It is also one of the prime androgen regulated miRNA in the prostate (Wang et al., 2008a, Ribas et al., 2009, Jalava et al., 2012); however, overexpression of miR-21 in castration-resistant cancers suggests that non-androgenic regulators can also increase miR-21 expression (Ribas and Lupold, 2010).

miR-125b is one of the more ambiguous oncogenic miRNAs in prostate cancer. The depletion of miR-125b in PC3 cells resulted in severe proliferation defects (Lee et al., 2005) and stable transfection of miR-125b into PC346C cells promoted their growth when xenografted (Shi et al., 2011). This androgen-regulated miRNA can promote cell proliferation by inhibiting the pro-apoptotic BAK1 protein, MUC-1 and ELF4EBP1 (Shi et al., 2007, Ozen et al., 2008, Rajabi et al., 2011). Nevertheless, the oncogenic role of miR-125b needs further substantiation as it has also been shown to be downregulated in prostate cancer (Porkka et al., 2007, Ozen et al., 2008, Tong et al., 2009, Sun et al., 2011). miR-125b can inhibit the expression of HER2 (Scott et al., 2007), which was previously shown to promote prostate carcinogenesis (Craft et al., 1999). These findings put miR-125b in the tumour-suppressor category. Evidence is also accumulating for the critical role of miR-125b in the regulation of epithelial stem cell differentiation (Shi et al., 2010, Cui et al., 2012, Wong et al., 2012, Wu et al., 2012). Any differences in expression of miR-125b levels in prostate cancer studies may therefore merely indicate differences in differentiation status of the samples analysed rather than an actual oncogenic role.

In summary, all these miRNAs need further evaluation in cells closer to patients (primary cells) and identification of all the potential targets in a cell-type specific manner in physiological miRNA doses to attest their precise role in prostate carcinogenesis.

III1.4.2 Tumour-suppressor miRNAs in prostate cancer

Several well-established tumour suppressor miRNAs such as miR-143, miR-145, and the miR-15a-16 cluster have also been implicated in prostate cancer.

miR-143 and miR-145 are probably the most studied miRNAs in relation to prostate cancer. These miRNAs are consistently down-regulated at all stages of prostate cancer, irrespective of the models used (Coppola et al., 2010, Szczyrba et al., 2010, Zaman et al., 2010, Leite et al., 2011). miR-143 underexpression can promote KRAS, cyclin D1, KLK10, and ERK signalling pathways to promote cell proliferation and migration (Clape et al., 2009, Xu et al., 2011a, White et al., 2012). Overexpression of miR-145 in PC3 and DU145 cells suppressed cell proliferation, migration and invasion through inhibition of FSCN1 and SWAP70 (Chiyomaru et al., 2011, Fuse et al., 2011). miR-145 transfection into PC3 cells can also induce apoptosis and G2/M cell cycle arrest by inhibiting TNFSF10 (Zaman et al., 2010). The major clinically relevant role of these two miRNAs is in the regulation of prostate cancer bone metastasis. Lower expression of these miRNAs was found in bone metastasis and forced overexpression of any one of these miRNAs reduced bone metastasis in PC3 prostate cancer mouse xenograft models (Peng et al., 2011, Watahiki et al., 2011). The increase in metastatic ability could be due to the initiation of epithelial to mesenchymal transition (EMT) and/or induction of a cancer stem-like phenotype (Peng et al., 2011, Huang et al., 2012). Promoter DNA methylation and, to a certain extent, P53 status can regulate the expression of these two miRNAs and hence, prostate carcinogenesis (Zaman et al., 2010, Suh et al., 2011). Thus, miR-143 and miR-145 could form a vital link between genetic and epigenetic alterations responsible for prostate carcinogenesis.

In addition to miR-143/-145 miRNAs, the miR-15a~16-1 cluster is also downregulated at almost all the stages of prostate cancer (Hassan et al., 2012). Bonci and colleagues were the first to show that miR-15a~16-1 expression is

downregulated in prostate cancer patients (Bonci et al., 2008). They also showed that miRNAs belonging to the miR-15a~16-1 cluster inhibit the expression of CCND1 and WNT3A and restoration of miR-15a~16-1 in LNCaP cells can induce apoptosis and growth arrest, whereas their inhibition in RWPE-1/2 cells imparts tumorigenic properties to these cells. The downregulation of this cluster could most likely be due to frequent homozygous deletion of 13q14 locus in a subset of prostate cancer patients, where the genes for miR-15a~16-1 cluster are located (Porkka et al., 2011). The miR-15a~16-1 cluster can also influence cell cycle through the regulation of CDK1/2 and cell proliferation through granulin in prostate cancer cell line models (Takeshita et al., 2010, Wang et al., 2010). The tumour-suppressor role of these miRNAs is further exemplified by the finding that systemic delivery of these miRNAs can significantly reduce tumour growth in a mouse PC3-M prostate cancer xenograft (Takeshita et al., 2010). Considering that miRNAs have cell-specific expression patterns and preferential function, it is unusual to find that the miR-15a~16-1 cluster could also be functionally important in prostate stroma. The lower expression of the miR-15a~16-1 cluster in prostate cancer-associated fibroblasts (CAFs) was shown to assist prostate cancer cell survival, proliferation, and migration, promoting FGF2-FGFR1 signalling (Musumeci et al., 2011). Thus, this cluster can coordinate between prostate cancer epithelial cells and CAFs to regulate prostate carcinogenesis.

Several additional miRNAs, such as miR-32 and the miR-106b-25-93 cluster have been proposed as oncomiRs and miR-101, miR-126*, miR-146a, miR-141, miR-200 family, miR-23, miR-330, miR-34, and miR-488 have been proposed as tumour-suppressor miRNAs. But the direct functional evidence for any of them is inconsistent in prostate cancer and needs further evaluation (Coppola et al., 2010, Pang et al., 2010, Hassan et al., 2012).

III1.4.3 The role of miRNAs in the process of epithelial to mesenchymal transition (EMT) in prostate cancer

Several recent studies have suggested that EMT may play a crucial role in cancer metastasis (Voulgari and Pintzas, 2009). Some investigators also believe that EMT can generate cancer stem-like cells from a differentiated progeny (Mani et al., 2008, Singh and Settleman, 2010). If this were true, then the knowledge of miRNA-driven regulation of EMT would be vital to understand cancer initiation and metastasis. The EMT process is characterised by loss of cell-cell junctions and epithelial markers, such as E-cadherin, ZO1, and occludin (Hay, 1995, Thiery, 2002). At the same time, cells acquire expression of mesenchymal markers such as vimentin, fibronectin, snail, slug, ZEB1/2, alpha-smooth muscle actin, beta-catenin, and various matrix metalloproteinases (MMPs) (Hay, 1995, Thiery, 2002). The process of EMT can be triggered by growth factors and cytokines, WNT and NOTCH signalling, stress, free radicals, hypoxia etc. (Voulgari and Pintzas, 2009). miRNAs that can alter any of these molecular pathways or processes can potentially promote or reverse the process of EMT.

Although several miRNAs can potentially regulate EMT, there is direct evidence for relatively few miRNAs in the regulation of EMT in prostate cancer. Peng et al, showed that upregulation of miR-143 and miR-145 can repress multiple mesenchymal markers while increasing epithelial markers in the PC3 and LNCaP cells (Peng et al., 2011). The restoration/overexpression of miR-205/miR-203 and miR-29b can also promote the epithelial phenotype over the mesenchymal phenotype by upregulating E-cadherin and downregulating ZEB2 in the PC3 and DU145 prostate cancer cell lines (Gandellini et al., 2009, Saini et al., 2011, Ru et al., 2012, Tucci et al., 2012). Similar downregulation of mesenchymal markers ZEB1/2 and Snail and upregulation of epithelial markers was also seen with downregulation of miR-200 and let-7 family members in the PC3 PDGF-D and $ARCaP_M$ cells (Kong

et al., 2009, Kong et al., 2010). These studies also showed that miR-143, miR-145, miR-205/miR-203, and miR-29b expression was downregulated and miR-200 and let-7 family was overexpressed in metastatic prostate cancer. A similar pattern of expression of miRNAs was also reported in various prospective normal *and* cancer stem cells (Peter, 2009, Wang et al., 2011b, Huang et al., 2012, Peng et al., 2012). These data suggest that miRNAs can be crucial for the regulation of prostate metastasis through EMT and may act to maintain the prostate cancer stem cell phenotype.

III1.4.4 miRNAs in prostate cancer stem cells

The tissue-specific expression and important role of miRNAs in lineage specification suggested the possibility of a distinct prostate cancer stem cell (CSC) specific miRNA expression profile. However, there is a very limited number of studies that have assessed prostate cancer stem cell miRNA expression in detail. One of the few miRNAs investigated for its specific role in the maintenance of prostate cancer stem cells is miR-34a (Liu et al., 2011). This investigation suggested that cancer stem cell-driven tumour growth and metastasis can be suppressed by miR-34a overexpression. They showed that miR-34a can inhibit CD44 expression in prostate CSCs derived from DU145 and LAPC4 prostate cancer cells. Using a similar model system, Saini and colleagues showed that miR-708 can also suppress CD44 expression and re-expression of miR-708 in prostate CSCs can lead to decreased tumorigenicity in vitro and in vivo, probably through direct inhibition of serine/threonine kinase AKT2 (Saini et al., 2012). Several other studies identified associations, rather than direct causal relationships between miRNAs and the prostate CSC-like phenotype. For example, let-7 overexpression can inhibit both prostate cancer cell proliferation and clonal expansion in vitro, and tumour regeneration in vivo using cell line models, without identifying any underlying mechanism (Liu et al., 2012). Over-expression of miR-101, miR-181a, miR-181b,

miR-200a, miR-200b, miR-200c, and miR-203 was also found to inhibit sphere formation by suppressing EZH2 in DU145 cells, suggesting their role in prostate CSC maintenance (Cao et al., 2011). Loss of miR-8/200 can potentiate NOTCH signalling in the PC3 prostate cancer cell line (Vallejo et al., 2011), which in turn can maintain prostate CSCs (Ceder et al., 2008). A modest reduction in both sphere formation ability and classical stem cell markers (such as CD133, CD44 and KLF4) was also seen after miR-143/145 overexpression in PC3 cells (Huang et al., 2012). Similar observations were also made in mouse models, where members of miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-203 were found to be specifically suppressed in the stem/basal ($Lin^{-}Sca\text{-}1^{+}CD49f^{high}$) cell compartment (Zhang et al., 2010). However, these associations may not guarantee the functional utility of these miRNAs. Furthermore, identification of just one or a few targets may not be enough to assess the full functional potential of miRNAs. Detection of miRNAs and the majority of their functional targets that are specific and critical for prostate CSC maintenance could bring about personalised, specific, and efficient management strategy for prostate cancer. In order to realise the true therapeutic potential of miRNAs, a more robust functional assessment in near-patient models, such as primary prostate epithelial cultures and in-vivo validation is essential.

III1.4.5 miRNAs as a biomarkers in prostate cancer

The prostate cancer field has long been suffering from a lack of specific and sensitive biomarkers for disease diagnosis and prognosis. Prostate specific antigen (PSA) is widely used, but its employment results in significant over-diagnosis and it is not an ideal predictor for therapy response and prognosis (Schroder et al., 2009, Andriole et al., 2012). Recent investigations have shown that the detection of circulating tumour cells (CTCs) could be a better biomarker for prostate cancer prognosis (Danila et al., 2007, Garcia et al., 2007, Scher et al., 2009). miRNAs are

relatively stable and can also be detected in CTCs, plasma, and serum (Schaefer et al., 2010b, Gordanpour et al., 2012). Bryant and colleagues recently demonstrated that the expression of miRNAs can be also be reliably detected in the urinary exosomes (Bryant et al., 2012). About 10 investigations have now assessed the suitability of relatively non-invasive detection of miRNAs for the prognosis of prostate cancer (Table III1.1). The standout miRNAs in these analyses are miR-141 and miR-375. The overexpression of these miRNAs can differentiate between normal and cancerous prostate, in addition to distinguishing aggressive cancers and CRPC from low risk prostate cancer (**Table III1.1**).

miRNA	**Sample origin**	**Expression in cancer** (compared to normal/BPH tissue)	**Reference**
miR-141	Human plasma	Up	(Mitchell et al., 2008)
	Human serum	Up	(Brase et al., 2011)
	Human plasma	Up	(Gonzales et al., 2011)
	Human plasma	Higher in metastatic vs. localized disease	(Yaman Agaoglu et al., 2011)
	TRAMP mouse serum	Up	(Selth et al., 2012)
	Human serum derived exosomes and microvescicles	Up in patients with metastasis than patients without metastasis	(Bryant et al., 2012)
	Human plasma	Up in CRPC	(Nguyen et al., 2012)
miR-375	Human serum	Up	(Brase et al., 2011)
	TRAMP mouse serum	Up	(Selth et al., 2012)
	Human serum derived exosomes and microvescicles	Up in patients with metastasis than patients without metastasis	(Bryant et al., 2012)
	Human plasma	Up in CRPC	(Nguyen et al., 2012)
miR-21	Human plasma	Up in cancer and in metastatic vs. localized disease	(Yaman Agaoglu et al., 2011)
	Human plasma	Up in patients with high risk CAPRA score	(Shen et al., 2012)

miR-30c	Human serum	Down in patients with high risk CAPRA score	(Moltzahn et al., 2011)
	Human serum	Down	(Chen et al., 2012b)
let-7c	Human serum	Down	(Chen et al., 2012b)
let-7e	Human serum	Down	(Chen et al., 2012b)
miR-20a	Human plasma	Up in patients with high risk CAPRA score	(Shen et al., 2012)
miR-24	Human serum	Down in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-26b	Human serum	Down in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-26a	Human serum	Up	(Mahn et al., 2011)
miR-93	Human serum	Up in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-106a	Human serum	Up in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-107	Prostate cells in urine	Up	(Bryant et al., 2012)
miR-221	Human plasma	Up in cancer and in metastatic vs. localized disease	(Yaman Agaoglu et al., 2011)
miR-223	Human serum	Down in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-298	TRAMP mouse serum	Up	(Selth et al., 2012)
miR-378*	Human plasma	Up in CRPC	(Nguyen et al., 2012)
miR-409-3p	Human plasma	Down in CRPC	(Nguyen et al., 2012)
miR-451	Human serum	Up in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-574-3p	Prostate cells in urine	Up	(Bryant et al., 2012)
miR-622	Human serum	Up	(Chen et al., 2012b)
miR-874	Human serum	Up in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-1207-5p	Human serum	Up in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-1274a	Human serum	Up in patients with high risk CAPRA score	(Moltzahn et al., 2011)
miR-1285	Human serum	Up	(Chen et al., 2012b)

Table III1.1: miRNAs secreted in body fluids, which can be used as diagnostic and/or prognostic biomarkers for prostate cancer.

However, the functions of these miRNAs and of CSC-specific miRNAs have not been investigated in detail. Sufficient information about the possible utility of miRNAs to predict/evaluate treatment response is also not available. Moreover, the overexpression/suppression of any of these miRNAs is not prostate cancer-specific. We also do not know whether miRNAs in Table III1.1 are expressed in prostate CSCs. If they are not, then recurrence caused by CSCs would be difficult to detect. Clearly, miRNAs could be the long-sought ‘ideal’ biomarkers for prostate cancer; but this needs further evaluation.

SECTION III: 2. Aims and objectives

mRNA levels profiling on hierarchical patient-derived prostate epithelial sub-populations have yielded a wealth of data, but no such resource exists for miRNA expression (Birnie et al., 2008, Shepherd et al., 2008). Previous miRNA microarray studies in cell lines have already suggested that prostate stem and differentiated cell have distinctly different miRNA expression profiles (Liu et al., 2012). This study in cell lines was restricted to cancer cell lines only, and a detailed miRNA expression profile of normal, BPH, and CRPC-derived sub-populations is not available. Previous microarray studies on primary tissue samples also suffered from wild variations in the expression profiles generated. Considering the enormous potential of miRNAs in developing therapeutic/diagnostic/prognostic strategies for the management of prostate cancer, establishment of consistent miRNA expression profile in patient-derived primary prostate epithelial subpopulations derived from normal, BPH, treatment naïve prostate cancers (PCa), and CRPCs was essential. Therefore, we decided to profile the expression pattern of miRNAs in stem cells, transit-amplifying cells, and committed basal cells enriched from PrEC (normal), BPH, PCa, and CRPC derived primary prostate epithelial cultures. The data obtained from such an investigation then could be paired with previous mRNA microarray profiles obtained on the similar hierarchical primary prostate epithelial sub-populations in our lab (Birnie et al., 2008) to ascertain genome-wide miRNA-mRNA alterations in prostate epithelial differentiation and prostate carcinogenesis. The output from this integrated dataset could be exploited to identify cancer stem cell directed therapeutic targets in addition to prognostic and diagnostic biomarkers.

SECTION III: 3. Results

The emerging role of miRNAs in cancer risk stratification, diagnosis, and prognosis prompted us to profile miRNA expression in cultured primary prostate epithelial sub-populations. Using Agilent v3 microarrays, expression of nearly 860 miRNAs was assessed in stem cells (SC), transit-amplifying cells (TA), and committed basal cells (CB) enriched from PrEC (n=1), benign (n=5), treatment naïve cancer (n=5), and castration resistant cancers (n=3). The array results were validated by qRT-PCR and prospective candidate miRNAs with potentially important roles in prostate stem cell differentiation were chosen. In addition, the miRNA expression data was integrated with previously generated mRNA levels data from our lab (Birnie et al., 2008) on a genome-wide scale, the first of its kind, to identify direct correlations between miRNA and target mRNAs expression in primary prostate epithelium. The entire investigation was performed in collaboration with Prof. Tapio Visakorpi from University of Tampere, Finland and Mr. Antti Ylipää from Tampere Technical University, Finland.

III3.1 Microarray for miRNA expression

III3.1.1 Microarray data quality control

The latest Agilent array design compatible with the local instruments was employed to assess expression profile of 866 miRNAs in normal and pathological primary prostate epithelial cultures. The microarray analysis was carried out on 100 ng of total RNA extracted from each of the 42 epithelial sub-populations. The assessment of Agilent internal quality control parameters and the distributions of summarized expression values for each sample ascertained the uniformity of labelling and hybridisation **(Figure III3.1)**.

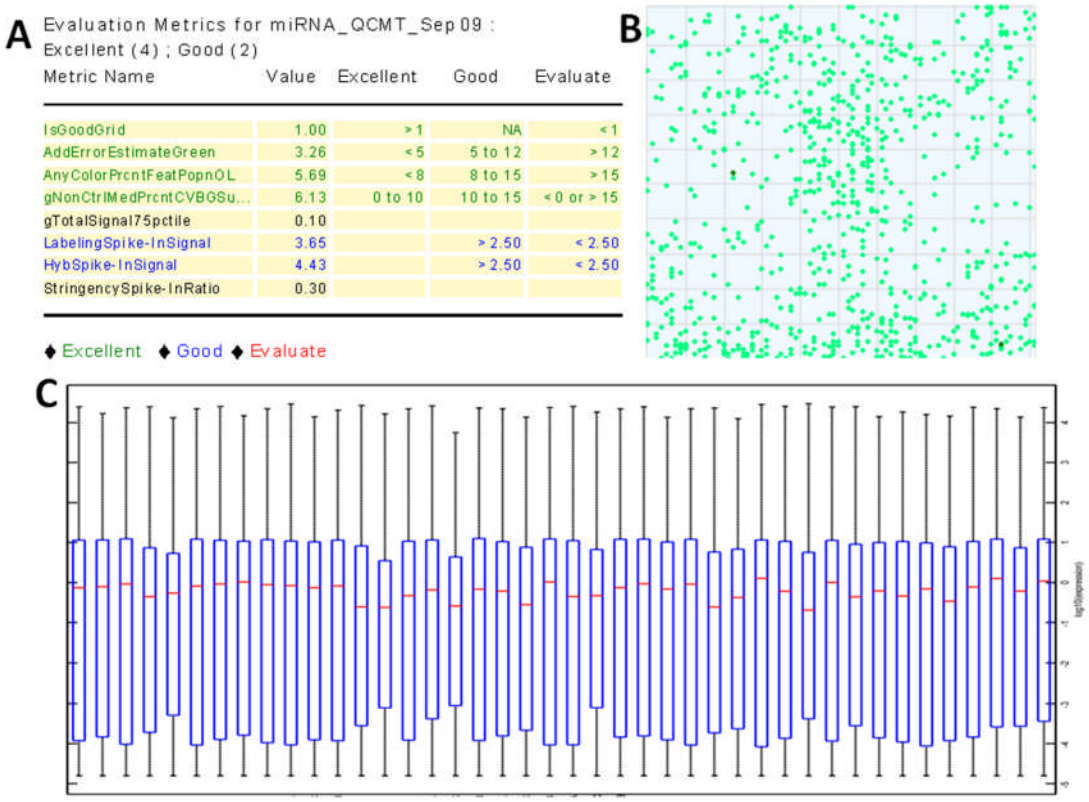


Figure III3.1: Quality assessment for miRNA microarray procedure. **A:** A report from Agilent's internal quality control program showing the parameters assessed fall in either excellent or good range. **B:** A virtual image of representative of the hybridised array chip. Each dot indicates hybridised miRNA probe. **C:** Boxplots created by *log*2 transformed probe intensity values that compare the probe intensity levels between the arrays of a dataset. Either end of the box represents the upper and lower quartile. The red line in the middle of the box represents the median (Fig.3. C: Prepared by Mr. Antti Ylipää).

III3.1.2 Heatmaps generated from microarray data

The raw microarray data was quantile normalised and RMA summarised (by Mr. Antti Ylipää). Lists were prepared that compared miRNA expression among various populations and ranked by p-values generated from paired two-tailed t-test and Wilcoxon rank sum test. The heatmaps generated from the non-normalised (**Figure III3.2**) and normalised (**Figure III3.3**) microarray output data showed two interesting phenomena suggestive of a principal role for miRNA in prostate epithelial differentiation: a distinct, cell type specific, miRNA expression pattern and global miRNA expression down-regulation during differentiation. The most undifferentiated cells (stem cells-SC) had much higher miRNA expression than the most differentiated cells in the analysis (committed basal cells-CB) (**Figure III3.2**). The miRNA expression pattern in transit-amplifying (TA) cells, which have functional characteristics intermediate between SC and CB, also had an intermediate miRNA expression pattern. In addition, stem cells, irrespective of their pathological status, had a higher total miRNA expression than the differentiated committed basal cells. Both of these patterns were suppressed in CRPC derived sub-populations (**Figure III3.2**), where the normal differentiation process is perturbed, again stressing the significant role of miRNAs in prostate epithelial differentiation.

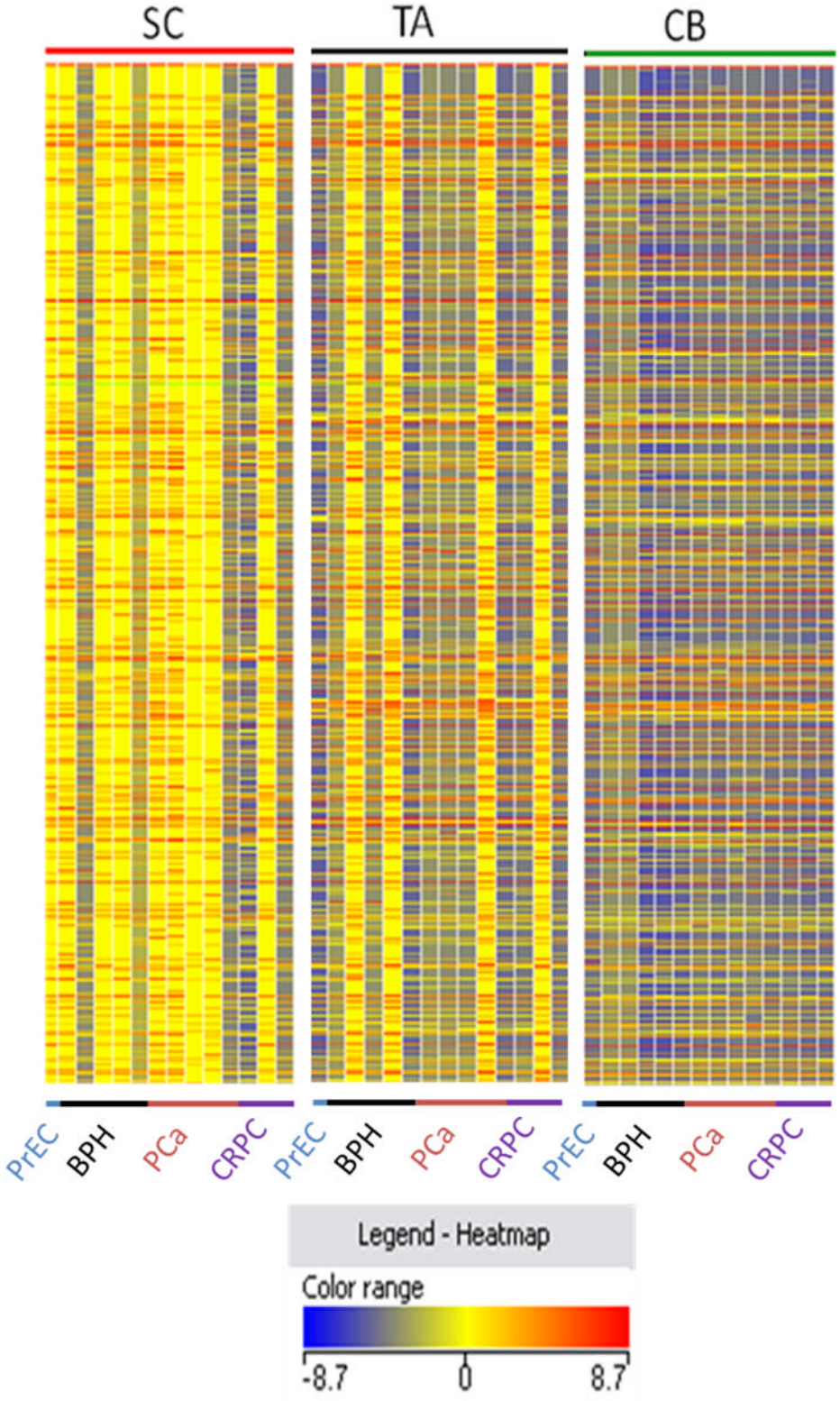


Figure III3.2: Supervised clustering of non-normalised expression of all the miRNAs represented as a heatmap**.** The 42 samples were clustered according to cell-type: stem cells (SC), transit-amplifying cells (TA), and committed basal cells (CB). In each cell cluster, cells are arranged as PrEC (n=1) first then BPH (n=5), PCa (n=5) and CRPC (n=3) at the end. The raw expression values were plotted to generate a heatmap using the Agilent software.

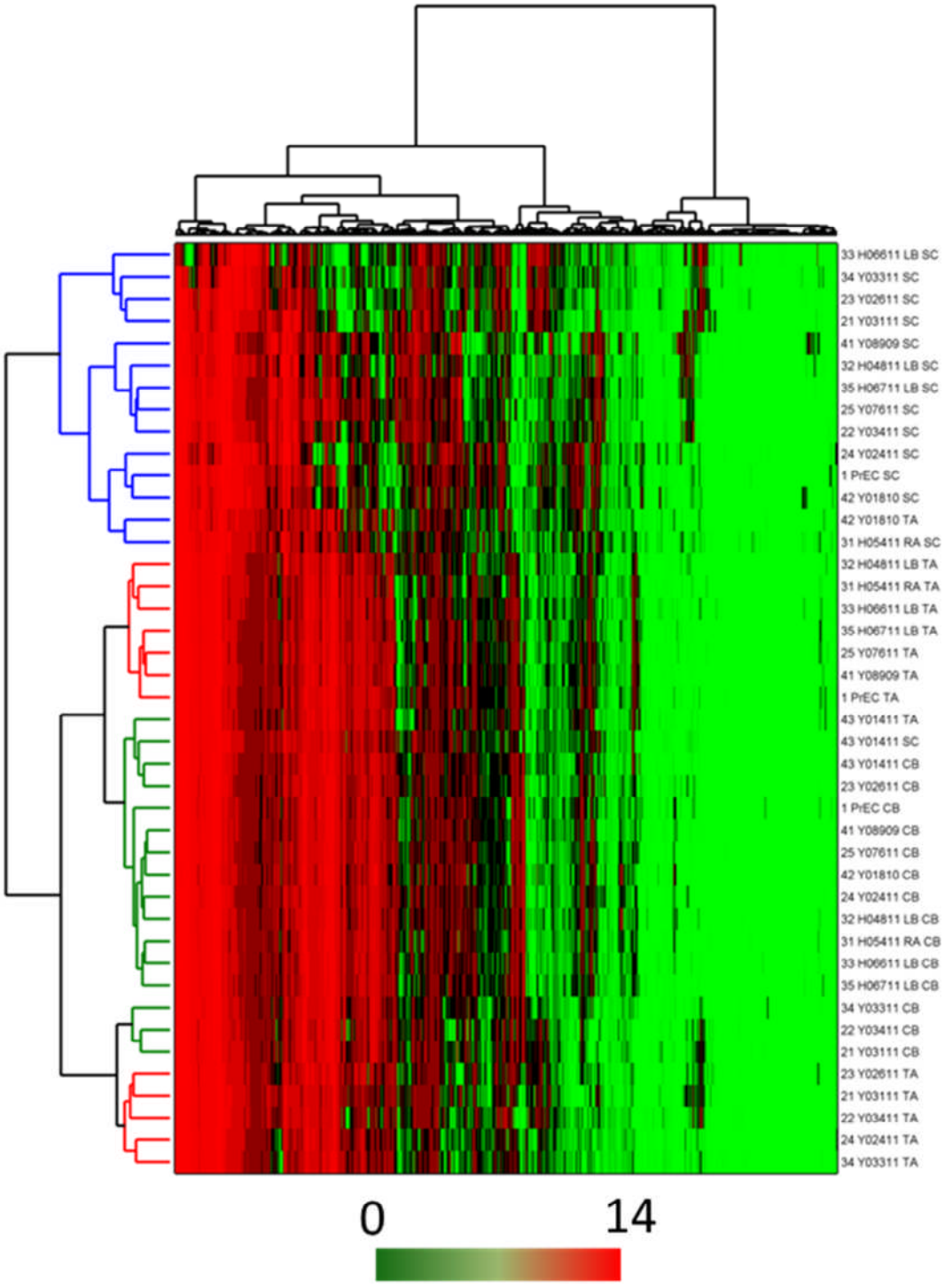


Figure III3.3: Unsupervised hierarchical clustering of miRNA microarray expression data after Robust Multi-array Average (RMA) normalisation. The raw output data was mapped to the latest genome database, zero or negative intensities were replaced with the lowest positive intensity values, the data were quantile normalized, and RMA summarized [By Mr. Antti Ylipää, from Tampere University of Technology, Tampere, Finland]. Prostate Samples are arranged from top to bottom and miRNAs horizontally. Each colour (blue/red/green) on the left hand side represents samples with similar miRNA expression profile.

III3.2 Validation of miRNA microarray data

We validated the array data by qRT-PCR using 5 randomly chosen samples (2 BPH and 3 cancers) from the samples that were used for the original arrays. The miRNAs for validation were chosen from miRNAs for which probes were already available in the lab and had some supporting literature for their role in prostate carcinogenesis. These miRNAs have different expression patterns, for example, let-7i, miR-29b, miR-143, miR145, and miR-32 were overexpressed with differentiation, mi-200c was overexpressed in TA cells compared to SC and CB cells, and miR-100 was not differentially expressed in any of the sub-populations in a microarray data. For normalisation of qRT-PCR data, RNU6b was used as an internal control, as it is the most commonly used internal control for miRNA qRT-PCR normalisation (Jung et al., 2009, Fridman et al., 2010, Snowdon et al., 2012). Although fold changes between the expressions of miRNAs in sub-populations were slightly different, the overall trend of expression was closely matched to that found in the array data (**Figure III3.4 and III3.5**). On confirmation of the accuracy, miRNA associated with differentiation and cancer in miRNA expression was assessed.

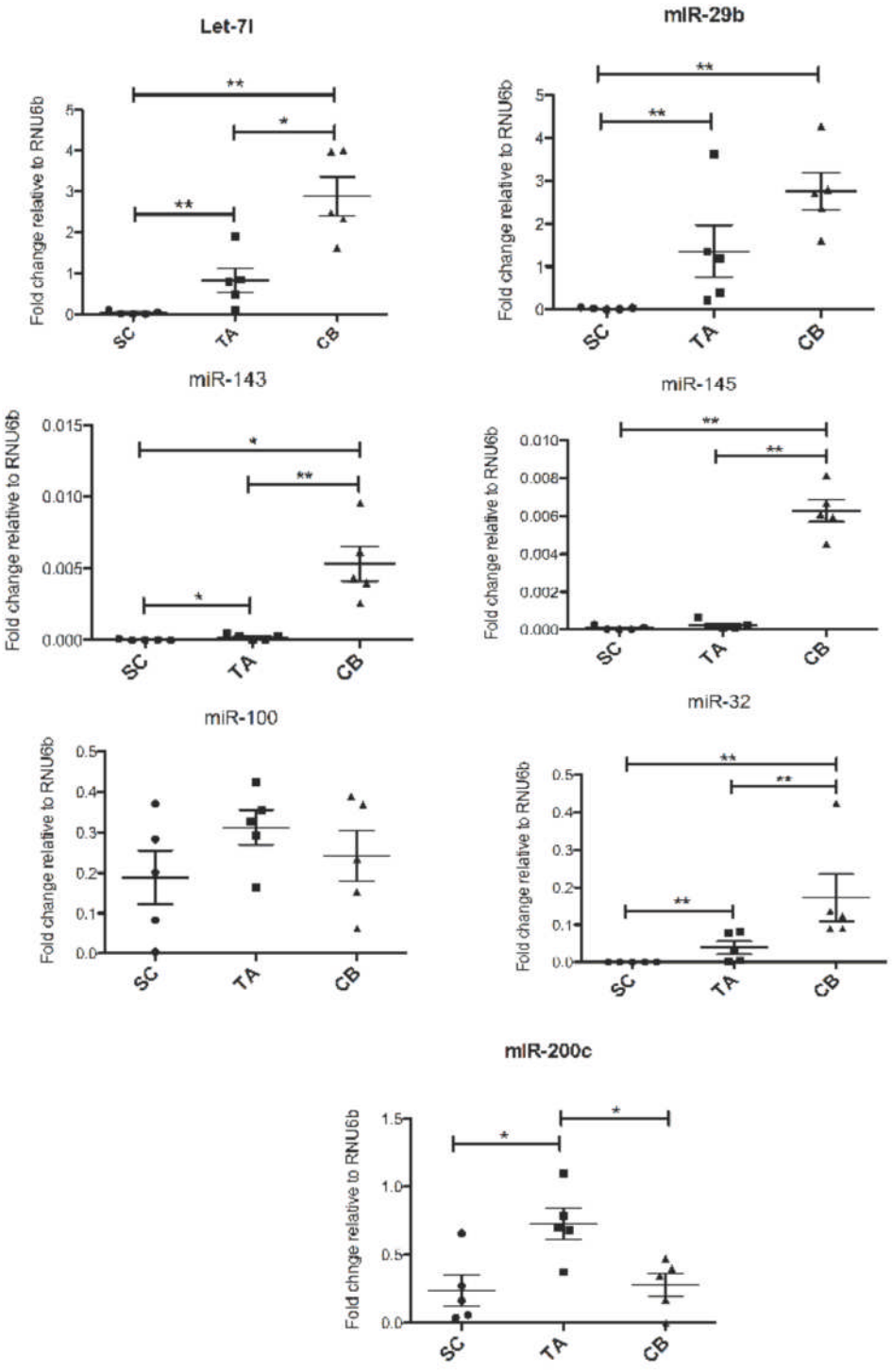


Figure III3.4: qRT-PCR validation of microarray results. The expression of 7 representative miRNAs qRT-PCR for 7 miRNAs tested in 5 randomly selected patient samples (2 BPH and 3 cancers). [* p<0.05, ** p<0.01 with paired two-tailed t-test and Wilcoxon rank sum test]

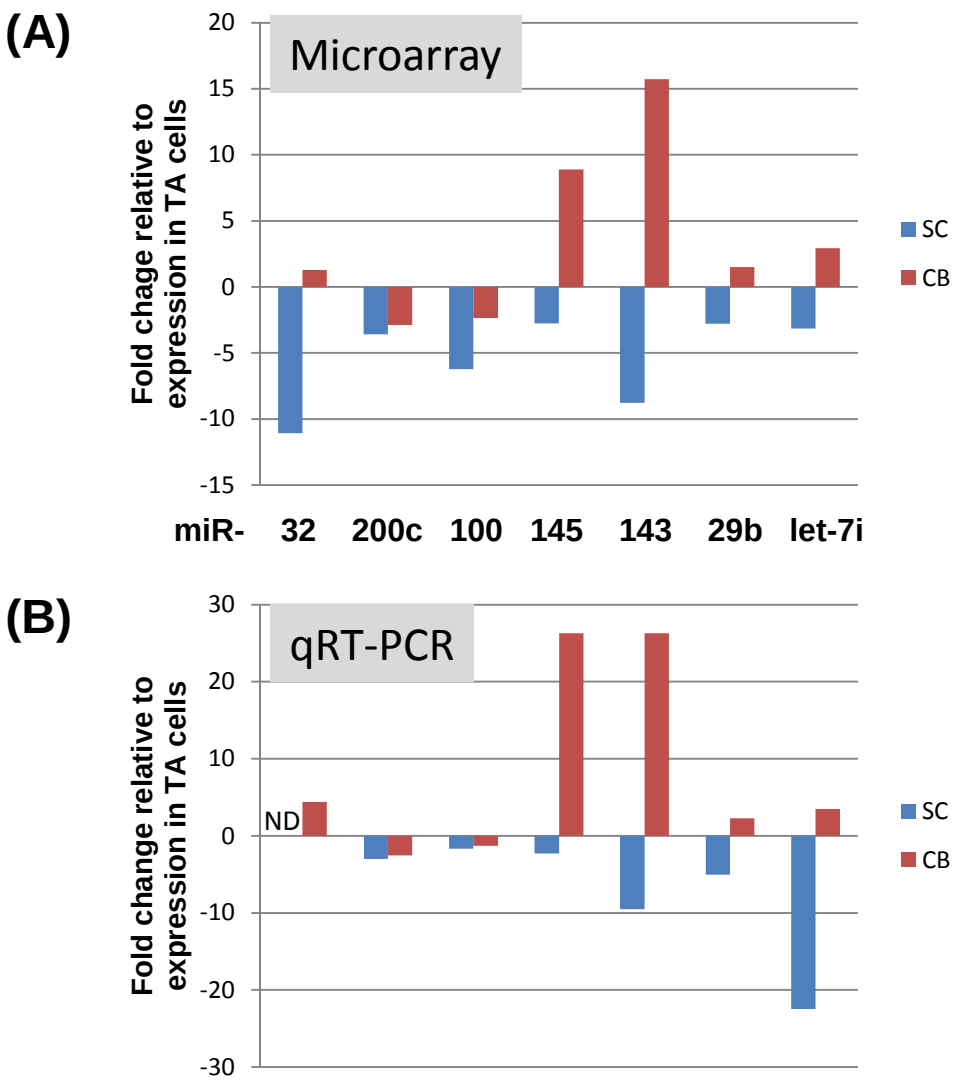


Figure III3.5: Comparison of miRNA expression in microarray data and in qRT-PCR validation. A: miRNA expression in in the microarray data B: miRNA expression in the qRT-PCR validation. The relative expression in SC and CB cells was plotted with reference to expression in TA cells. ND: not detectable.

III3.3 Prostate stem cell miRNA signature

We have identified 135 miRNAs that had detectable expression (>20 arbitrary units) and showed a greater than 3-fold change in expression between stem cells and differentiated committed basal cells (Heatmap **Figure III3.6**). All these miRNAs were significantly differentially expressed ($p<0.01$) with paired two-tailed t-test and Wilcoxon rank sum test. The 3-fold difference threshold was chosen to shorten the list of differentially expressed miRNAs between SC and CB populations (combined from all the pathological states) from 459 to 135. These differentially expressed miRNAs are listed in **Appendix 2 and 3**.

Some of these miRNAs have been implicated in the maintenance of wide variety of stem cells (e.g. let-7 family) (Liu et al., 2012), but some others had no known association with epithelial stem cell behaviour. Some of these miRNAs could represent novel and prostate-specific stem cell regulating miRNAs. Identification of direct mRNA targets for all the differentially expressed miRNAs in our analysis can link the miRNA with the known and novel genetic drivers of prostate stem cell behaviour.

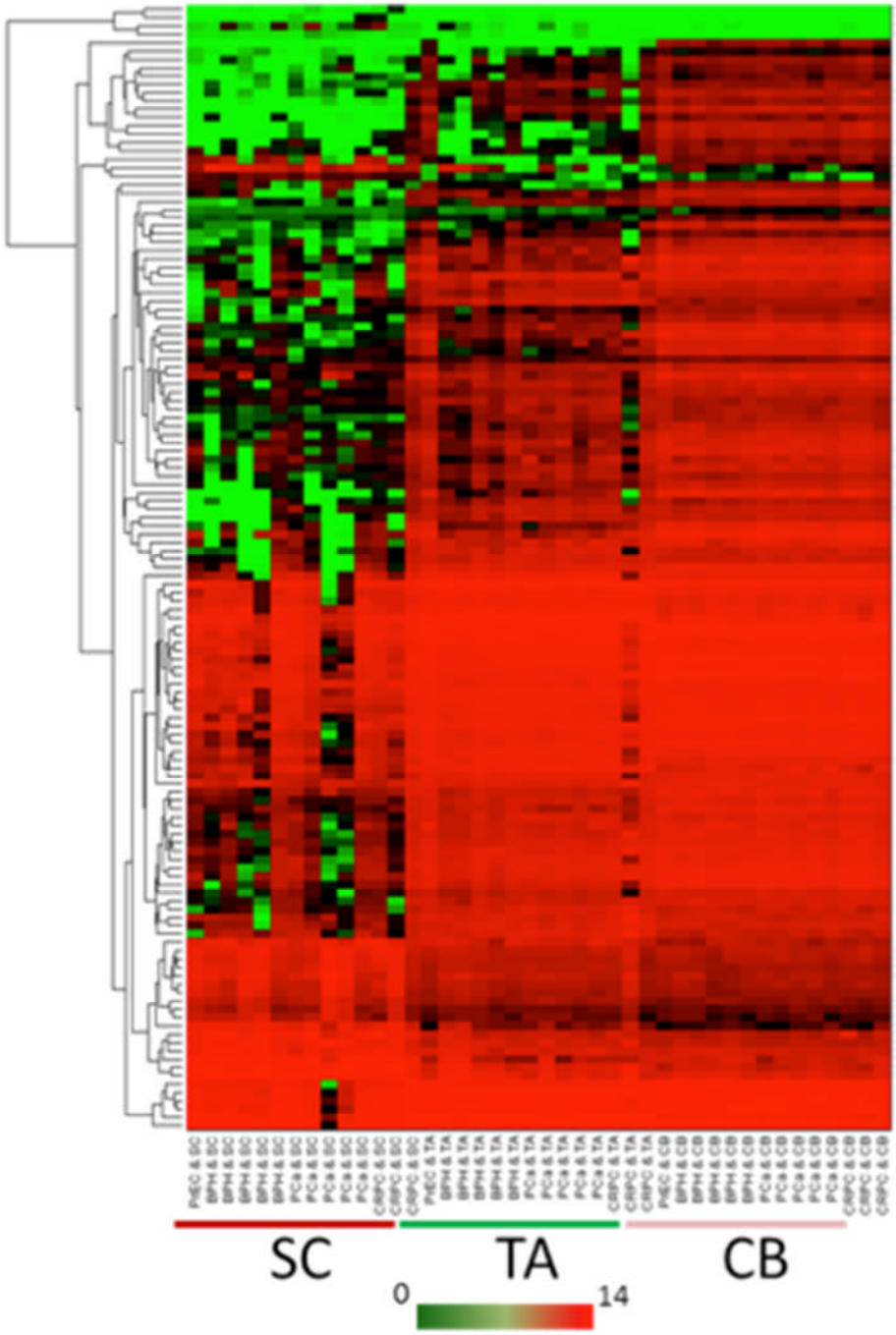


Figure III3.6: Supervised clustering of differentially expressed miRNAs in prostate epithelial sub-populations after Robust Multi-array Average (RMA) normalisation. The normalised expression of miRNAs in three different sub-populations (stem cells –SC, transit amplifying cells-TA, and committed basal cells-CB) was compared with each other. 135 miRNAs showed more than 3-fold difference in stem cells and committed basal cell expression with $p<0.05$ with Wilcoxon rank sum test and paired two-tailed t-test. These miRNAs are represented in this heatmap. Prostate Samples are arranged horizontally and miRNAs from top to bottom. [By Mr. Antti Ylipää, from Tampere University of Technology, Tampere, Finland].

III3.4 miRNAs associated with prostate cancer

A comparison was made to identify miRNAs that specifically characterised treatment naïve prostate cancer (PCa) and CRPC. The average miRNA expression of all the sub-populations enriched from BPH was compared with the average miRNA expression of all the sub-populations enriched from PCa or CRPC. The paired two-tailed t-test and Wilcoxon rank sum test were applied to determine significance. It should be remembered that the comparison in this study involves assessment of differential miRNA expression in the *basal* compartment of the respective pathology, as luminal cells do not grow in culture. Hence, the results obtained in our analysis were not directly comparable with the previously published whole unfractionated PCa and BPH sample comparisons (Coppola et al., 2010, Martens-Uzunova et al., 2012). Even if when we assessed this restricted (but probably therapeutically more relevant) sub-set of cells, we identified 7 miRNAs that were significantly differentially expressed in PCa compared to BPH (**Table III3.1**) and 16 miRNAs were significantly differentially expressed in CRPCs compared to PCa (**Table III3.2**).

miRNA	**PCa/BPH**
Upregulated	
hsa-miR-411*	4.71
hsa-miR-886-3p	3.93
hsa-miR-629	2.91
hsa-miR-1208	2.88
hsa-miR-1271	1.75
hsa-miR-423-3p	1.57
Downregulated	
hsa-miR-299-5p	-2.42

Table III3.1: Differentially expressed miRNAs in treatment naïve prostate cancer (PCa) compared to benign prostatic hyperplasia (BPH). Each fold change is statistically significant with $p<0.05$ using paired two-tailed t-test and Wilcoxon rank sum test.

miRNA	CRPC/PCa	miRNA	CRPC/PCa
Upregulated		**Downregulated**	
hsa-miR-146a	5.09	hsa-miR-1247	-34.54
hsa-miR-521	4.14	hsa-miR-125b-2*	-3.93
hsa-miR-576-5p	3.26	hsa-miR-375	-3.45
hsa-miR-302c	3.09	hsa-miR-203	-3.23
hsa-miR-924	2.88	hsa-miR-886-3p	-2.76
hsa-miR-520g	2.55	hsa-miR-193a-5p	-2.22
hsa-miR-520f	2.54	hsa-miR-200c*	-2.14
hsa-miR-221	1.62	hsa-miR-200b*	-1.71

Table III3.2: Differentially expressed miRNAs in castration resistant prostate cancer (CRPC) compared to treatment naïve prostate cancer (PCa). Each fold change is statistically significant with $p<0.05$ using paired two-tailed t-test and Wilcoxon rank sum test.

III3.5 Prostate cancer stem cell miRNA signature

Recently, the interest in identifying microRNA that are specifically deregulated in cancer stem cells has grown exponentially, as these miRNAs can be readily exploited for diagnostic/prognostic/therapeutic purposes. In order to identify a cancer stem cell specific miRNA signature, we first identified two sets of miRNAs: (i) miRNAs that were significantly differentially expressed between BPH-derived stem cells (SC) and differentiated cells and (ii) miRNAs that were significantly differentially expressed between treatment naïve cancer-derived stem cells (CSC) and differentiated cells (with $p<0.05$ using paired two-tailed t-test and Wilcoxon rank sum test). The miRNAs significantly differentially expressed in CSCs compared to nSCs were then identified (with $p<0.01$ using paired two-tailed t-test). In effect, the miRNAs listed in **Table III3.3** are either over- or under-expressed in cancer stem cells compared to normal or BPH stem cells, and at the same time, differentially expressed in cancer stem vs. cancer differentiated cells.

miRNA	CSC/SC	Comment
UPREGULATED		
miR-323-3p	161.31	No previous known relationship with cancer/stem cell regulation
miR-411	27.98	Proposed serum biomarker for breast cancer (van Schooneveld et al., 2012)
miR-33a	14.74	Cell cycle regulation (via cyclins and CDKs), and Involved in lipid metabolism, insulin signalling, and development of metabolic syndrome (Ibrahim et al., 2011, Rayner et al., 2011, Cirera-Salinas et al., 2012, Wijesekara et al., 2012)
miR-532-3p	10.60	Implicated in neuronal stem cell differentiation (Hamada et al., 2012)
miR-181a-2	8.65	Proposed therapeutic/prognostic role in gastric, head & neck, neural, skin, pancreatic and haematopoietic cancers (Seoudi et al., 2012)
miR-1271	3.03	Up-regulated in head & neck cancers (Nurul-Syakima et al., 2011)
miR-487b	2.68	Proposed biomarker for relapse in neuroblastoma (Gattolliat et al., 2011)
DOWNREGULATED		
miR-302c	-5.97	Regulate cell cycle and DNA damage response, prognostic and therapeutic role in breast cancer (Yoshimoto et al., 2011, Dolezalova et al., 2012)
miR-1181	-4.67	No previous known relationship with cancer/stem cell regulation
miR-519c-3p	-4.40	Associated with rectal cancer prognosis (Kheirelseid et al., 2012)
miR-574-5p	-2.41	Early stage biomarker for non-small cell lung cancer, chromatin modifier, and negatively regulate WNT signalling to promote colorectal cancer (Foss et al., 2011, Ji et al., 2012, Meyers-Needham et al., 2012)

Table III3.3: MicroRNA signature of prostate cancer stem cells: Table shows most highly over expressed and repressed miRNAs in prostate CSCs compared to BPH-derived SCs. All changes were statistically significant ($p<0.01$ with paired two-tailed t-test)

III3.6 Identification of candidate miRNAs, which can regulate prostate stem cell differentiation

In order to complement the transcriptional regulation of prostate stem cell differentiation analysis, we decided to identify miRNAs that can regulate prostate stem cell differentiation. We decided to choose 4 candidate miRNAs that can

regulate prostate epithelial stem cell differentiation. The criteria for this selection included:

- 2 miRNAs, which are significantly overexpressed in prostate epithelial stem cells (in all PrEC, BPH and PCa-derived stem cells) compared to CB cells.
- 2 miRNAs, which are significantly repressed in prostate epithelial stem cells (in all PrEC, BPH and PCa-derived stem cells) compared to CB cells.
- One miRNA from each of the above two groups should have a relatively well-established role in epithelial differentiation.
- The other miRNA from each of the two groups was chosen purely on the speculative basis. The selection was aided by the predicted targets of the miRNAs.

Based on these criteria, we chose miR-10a, miR-125b, miR-494, and miR-766 as candidate miRNAs, which could regulate primary prostate epithelial stem cell differentiation. Two of the miRNAs (miR-125b and miR-10a) were repressed in the stem cell compartment enriched from all the samples (normal, BPH, and treatment naïve cancer) and other two (miR-494 and miR-766) were upregulated in stem cell fraction. miR-125b was previously found to be significantly over-expressed during mammalian neuronal and retinal epithelial differentiation (Smirnova et al., 2005, Ferretti et al., 2008, Le et al., 2009). Concurrently, it was down-regulated in undifferentiated cutaneous melanomas (Glud et al., 2010) and hepatoblastomas (Cairo et al., 2010), and indeed, specifically in glioma stem cells (Shi et al., 2010). miR-125b also exhibited androgen dependent up-regulation in a differentiated prostate cancer cell line (Shi et al., 2007). These literature reports indirectly suggest that the down-regulation of miR-125b is necessary for maintaining stem cell self-renewal and quiescence by allowing expression of its targets, such as MYC, p53, and p21. On the other hand, miR-494 was expressed abundantly in the stem cell sub-population. Phosphatase and tensin homolog (PTEN) is one of the principal

target mRNAs inhibited by miR-494 (Yang et al., 2008, Liu et al., 2010b). It has been reported that inhibition of PTEN is necessary to maintain prostate stem cell self-renewal and their colony forming efficiency through prolonged activation of PI3/Akt pathway (Dubrovska et al., 2009, Hill and Wu, 2009). Over-expression of PTEN has also been linked with prostate cancer development and metastasis in mouse models (Wang et al., 2003). Therefore, we have decided to take miR-125b and miR-494 forward to assess their precise role in prostate stem cell differentiation. miR-10a and miR-766 were speculatively chosen on the basis of their predicted mRNA targets, such as TEAD, CEACAM6, and S100p (CEACAM6 and S100p are candidates genes investigated in SECTION I for their role in prostate stem cell differentiation, and TEAD has a binding site on the promoters of all the candidate genes under study for transcriptional regulation of prostate stem cell differentiation). The expression of these candidate miRNAs was then confirmed in 4 BPH and 4 PCa samples (**Figure III3.7**). These samples were different from those used to establish expression by microarray analysis.

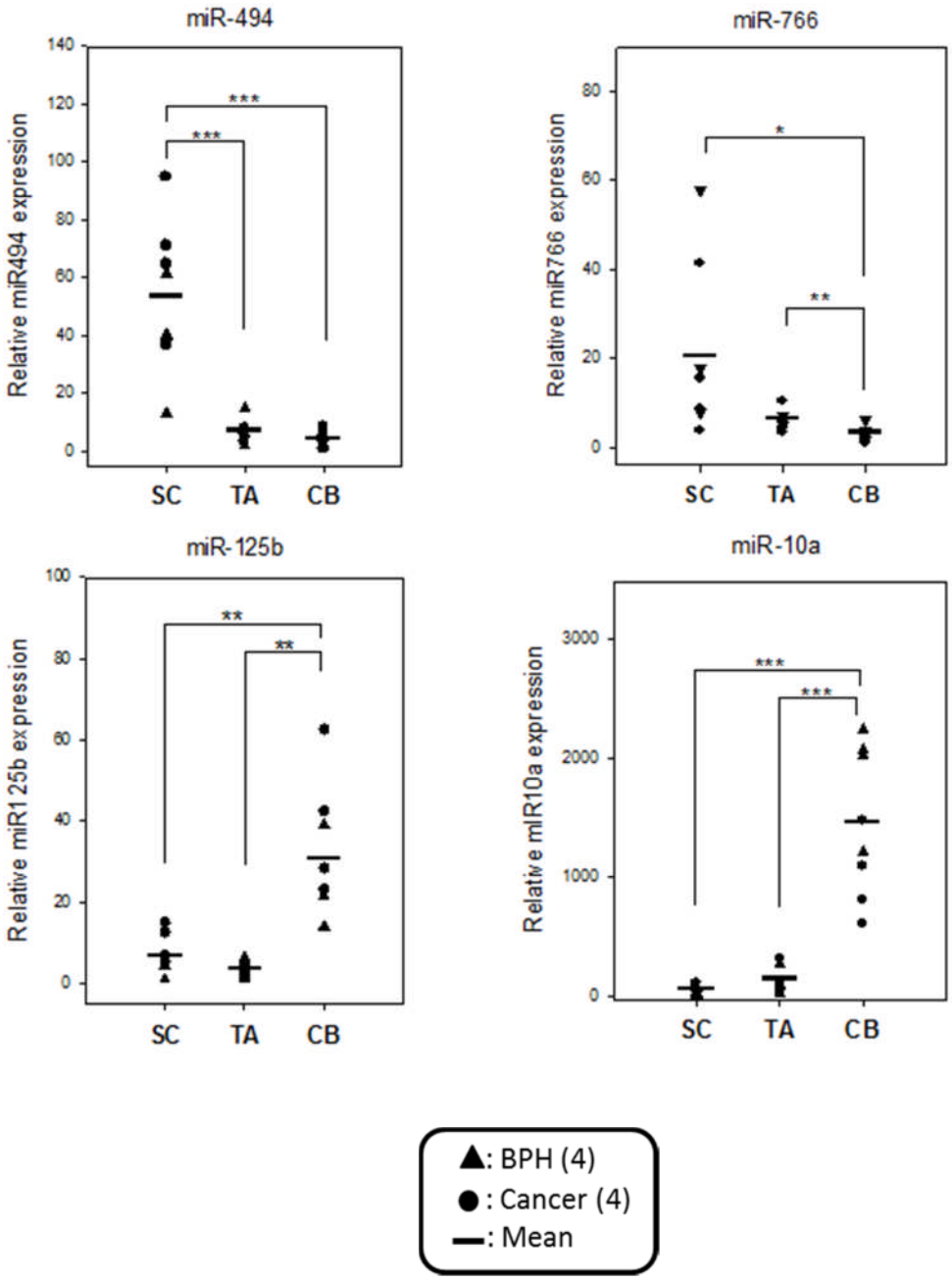


Figure III3.7: qRT-PCR analysis of miR-494, miR-766, miR-125b, and miR-10a in prostate epithelial sub-populations enriched from BPH and treatment naïve prostate cancer derived epithelial cultures. *p<0.05, **p<0.01, ***p<0.001 with two tailed t-test.

III3.7 miRNA-mRNA microarray Data integration

miRNAs influence multiple genes and hence, multiple signalling pathways. Determination of all these interactions is essential to understand the complete spectrum of miRNA effects. We therefore decided to integrate miRNA and mRNA microarray data, to identify genome-wide correlations between miRNAs and their respective target mRNAs in similar prostate epithelial samples, across both differentiation and carcinogenesis. Taking advantage of an already available mRNA microarray analysis performed in our lab (Birnie et al., 2008), we integrated the mRNA and miRNA microarray data results. The majority of mRNAs targeted by miRNAs showed a small but consistent reduction in steady-state levels due to mRNA destabilisation and degradation (Baek et al., 2008, Guo et al., 2010). Any negative correlation between miRNA expression and changes in the expression of their predicted mRNA targets could therefore identify direct miRNA functional effects. The pathways and processes which could be primarily regulated by miRNAs were then be detected by pathway and gene ontology analysis on all of the significantly correlated mRNA targets. The following parameters were set to obtain unbiased results:

- Filter out mRNA and miRNA that are not expressed in our datasets (348 miRNAs).
- Filter out those miRNAs that do not change between the subsets that are being compared.
- The mRNA targets of all the differentially expressed miRNAs in stem vs. committed basal cells with $p<0.05$ (paired two-tailed t-test and Wilcoxon rank sum test) were determined using the 5 prediction algorithms (miRanda, miRDB, RNA2, miRWalk, and TargetScan).
- Only the common mRNA targets predicted by all of the 5 prediction programs were then analysed for gene ontology and pathway analysis

- If there are no or very few common predicted targets, then choose the first 25 targets that have highest prediction values.
- From the chosen targets, filter out those targets that are not expressed in our mRNA microarray.
- Make a list of miRNAs and their targets (positive and negative correlation) that show a significant correlation.
- Perform GO and pathway analysis along with Gene Set Enrichment Analysis (GSEA).
- Carry out this analysis for SC vs. CB.

Mr. Antti Ylipää performed this analysis. In this analysis, we found that a significant number of miRNA targets were correspondingly downregulated in the prostate epithelial sub-populations. This strong correlation between miRNA and mRNA levels suggested that miRNAs may have a heavy influence on the regulation of several prostate epithelial differentiation related pathways. The pathway and GO analysis performed on correlated mRNA targets of differentially expressed miRNAs identified that cell cycle and epithelial-stromal (niche) related pathways could be mainly under miRNA regulation (**Table III3.4**).

	Pathway	P value
1	Mitotic cell cycle	7.79E-07
2	Cell cycle process	9.12E-06
3	Aurora B signaling	1.79E-05
4	Establishment of protein localization	5.18E-05
5	Signaling by Aurora kinases	5.55E-05
6	Protein localization	9.22E-05
7	Intracellular protein transport	0.000158
8	Cell cycle phase	0.000181
9	Protein import	0.000186
10	Endothelins	0.000192
11	Role of Calcineurin-dependent NFAT signaling in lymphocytes	0.000213
12	IL1-mediated signaling events	0.000218
13	Cell cycle	0.000221
14	Protein transport	0.000224
15	Macromolecule localization	0.000235
16	Class I PI3K signaling events	0.000247
17	Protein targeting	0.000267
18	Establishment of cellular localization	0.000325
19	Cell Cycle: Mitotic	0.00034
20	TRAIL signaling pathway	0.00034
21	IFN-gamma pathway	0.000376
22	Regulation of i-κB kinase NF-κB cascade	0.000392
23	Actin filament based process	0.000442
24	Cellular localization	0.000465
25	Cytoskeleton organization and biogenesis	0.000476
26	TNF alpha/NF-kB	0.000648
27	TGF-beta receptor signaling	0.001613
28	Regulation of nuclear SMAD2/3 signaling	0.001613
29	BMP receptor signaling	0.00241
30	G1/S Transition	0.004429
31	$\alpha_6\beta_4$ Integrin Signaling Pathway	0.005445

Table III3.4: Pathways principally regulated by miRNAs during primary prostate epithelial differentiation. mRNA targets of miRNAs, which change significantly and consistently during differentiation were determined using 5 prediction algorithms. The corresponding negative expression of these predicted targets was assessed in mRNA microarray data. The gene ontology and pathway analysis was performed on the target mRNAs that significantly correlated to the corresponding miRNA expression.

III3.8 Identification of a prostate specific miRNA

Finally, we wanted to investigate whether any of the miRNA in our analysis is preferentially expressed in the prostate epithelium. Such miRNA may then be employed in prostate-specific therapeutic and diagnostic strategies. miR-720 had the highest expression levels among all the miRNAs tested in our microarrays and, at the time of investigation, no other miRNA microarray analysis had demonstrated high miR-720 expression in any other tissue. So, we hypothesised that miR-720 could be a prostate specific miRNA. To test this, miR-720 expression was assessed in a panel of commercially available 20 normal human tissues from Lonza (**Figure III3.8**). However this analysis showed that miR-720 is not a prostate specific miRNA and its expression was higher in 16 other tissues than in prostate. Previous studies did not identify miR-720 expression in any tissue, because it was not represented on the older version of miRNA platforms.

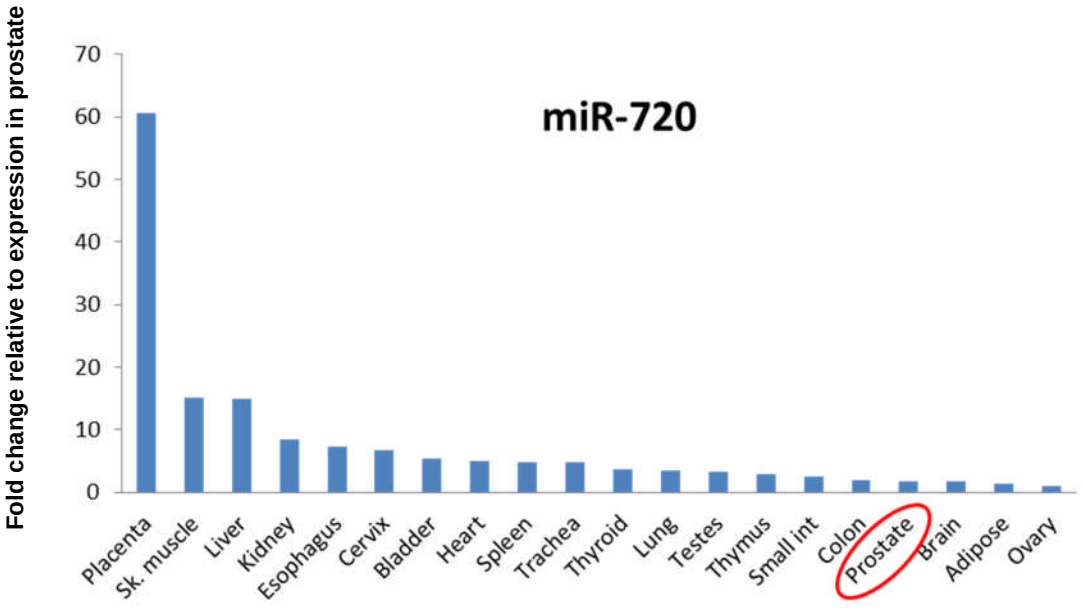


Figure III3.8: miR-720 expression in a panel of 20 human primary tissues. miR-720 expression was assessed by qRT-PCR. The expression was normalised to RNU6b and then plotted with reference to the expression in the prostate.

miR-720 also showed the least variability among all the samples tested in microarray analysis (**Appendix 4**: list of miRNAs with high expression and minimal variance) Together with high expression, a miRNA with the least variation across samples can be a good candidate as an internal control (or reference miRNA) for microRNA qRT-PCR studies. The small nucleolar RNAs, such as RNU6b, commonly used for this purpose have different kinetics and isolation efficiency compared to miRNAs (Peltier and Latham, 2008, Jung et al., 2010). The miRNAs are also considerably more stable than small nucleolar RNAs (Jung et al., 2010). So, we assessed the variability of miR-720 to that of RNU6b by comparing the variability of these small RNAs with respect to a standard curve and by calculating variability with the formula: std. dev./average *100 (**Figure III3.9 and Table III3.5**). First, the expression of miR-720 was about 15 fold higher than that of RNU6b. The higher expression of any internal control miRNA is beneficial to prepare a standard curve and in deltaCt method of the qRT-PCR analysis (Livak and Schmittgen, 2001). Secondly, the variability of miR-720 was slightly lower in all the sub-populations compared to that of RNU6b. The changes in the expression of RNU6b and miR-720 were also assessed when primary prostate epithelial cultures were transfected with hTERT siRNA. The expression of RNU6b and miR-720 did not change significantly after transfection (**Figure III3.10**). These findings suggest that miR-720 could be a better internal control miRNA for miRNA qRT-PCR analysis on primary prostate epithelial sub-populations. Comparisons could also be made with RNU6, but it has not been used extensively in prostate cancer miRNA studies.

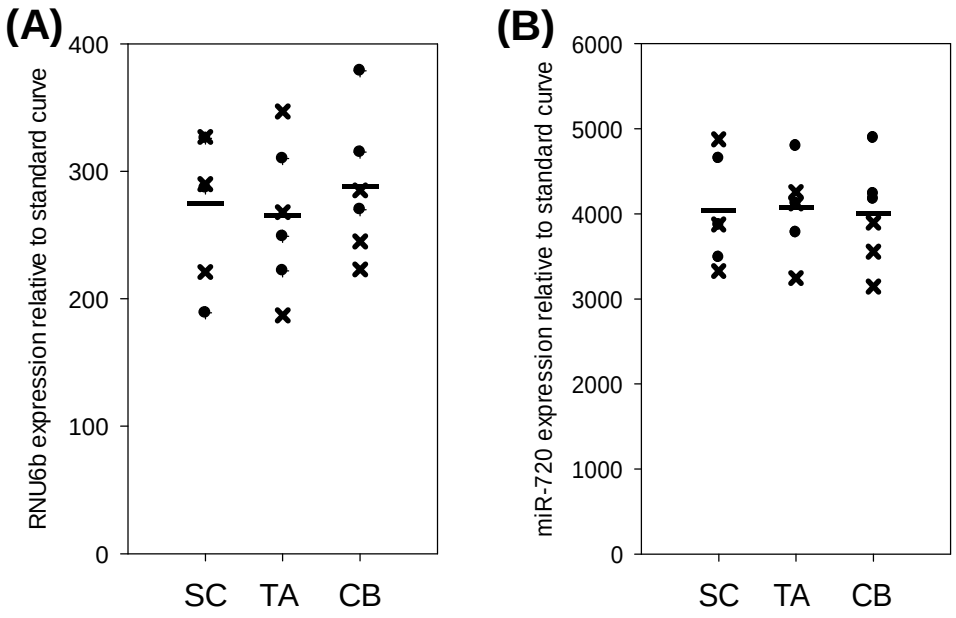


Figure III3.9: Comparison of variability in RNU6b and miR-720 expression in primary prostate epithelial sub-populations. A: RNU6b expression in primary prostate epithelial sub-populations enriched from BPH (●) and PCa (**x**) cultures, as determined by qRT-PCR. B: miR-720 expression in primary prostate epithelial sub-populations enriched from BPH (●) and PCa (**x**) cultures, as determined by qRT-PCR. The expression was plotted relative to line standard curve produced in PC3 prostate cancer cells. N=3 for BPH and N=3 for PCa.

	RNU6b variability (std. dev./average *100)	miR-720 variability (std. dev./average *100)
Stem cells	18.87	14.15
Transit amplifying cells	20.13	11.62
Committed basal cells	17.70	13.83

Table III3.5: Variability of RNU6b and miR-720 in primary prostate epithelial subpopulations.

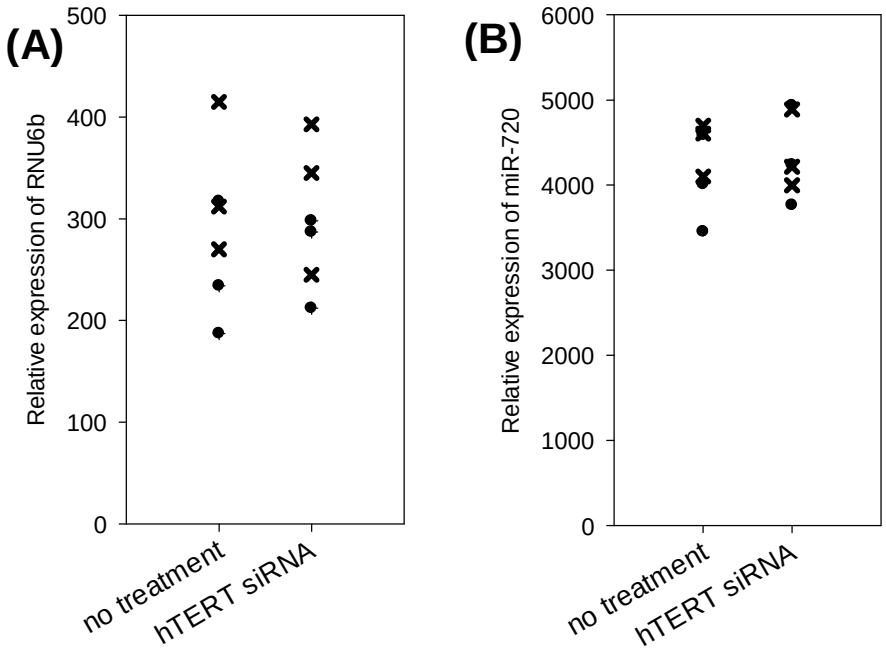


Figure III3.10: Changes in RNU6b and miR-720 expression after siRNA transfection. A: RNU6b expression in primary prostate epithelial BPH (●) and PCa (**x**) cultures, as determined by qRT-PCR. B: miR-720 expression in primary prostate epithelial BPH (●) and PCa (**x**) cultures, as determined by qRT-PCR. The expression was plotted relative to line standard curve produced in PC3 prostate cancer cells.

In summary, the miRNA expression analysis showed that the impact of differentiation on miRNA expression pattern was more robust compared to carcinogenesis. Based on this analysis, we have identified miR-125b, miR10a, miR-494, and miR-766 as potential target miRNAs, which may be essential for prostate stem cell differentiation. We have also established miR-720 as a better internal control for miRNA qRT-PCR normalisation in our system. The miRNA-mRNA genome-wide integrative database has further increased our understanding of gene regulatory networks in the prostate. Functional characterisation of miRNAs specifically deregulated in PCa and CRPC and their target pathways as determined by the miRNA-mRNA integrative database could identify novel management strategies for prostate cancer treatment.

SECTION III: 4. Discussion

In this study, primary prostate epithelial culture-derived homogeneous cellular sub-populations were employed to produce an expression profile of 866 human miRNAs in the stem cells and their differentiated progeny (TA and CB cells). We have now generated a consistent and statistically significant dataset, in which we can compare expression levels, disease association and differentiation driven control of miRNA expression in the patient-derived prostate tissue.

III4.1 miRNAs and differentiation

Numerous previous studies indicate that miRNAs perform important roles in cellular differentiation. Mice lacking the miRNA processing enzyme Dicer suffer developmental arrest during gastrulation (Bernstein et al., 2003), whereas haplo-insufficiency of Dicer results in increased tumorigenicity. Conditional Dicer knockout mouse embryonic stem cells also displayed severe defects in differentiation, both in vitro and in vivo (Kanellopoulou et al., 2005). Furthermore, mammalian stem cells and their differentiated progeny revealed notably discrete miRNA expression patterns (Chen et al., 2004, Krichevsky et al., 2006, Goff et al., 2008, Hildebrand et al., 2011). These findings together with reports illustrating induction of differentiation or dedifferentiation (iPS cell generation) by specific miRNAs, strongly indicate that miRNAs are critical regulators of stem cell differentiation (Lin et al., 2008, Tay et al., 2008, Li et al., 2011, Yi and Fuchs, 2011) suggesting that miRNA expression is very much cell-type dependent. We therefore carried out miRNA profiling in individual prostate epithelial sub-populations, rather than analysing the whole tissue mass (which contains all the epithelial sub-populations, stroma, immune infiltrate and endothelial cells).

The latest (at the time of investigation) microarray chips compatible with available processing platform were used to analyse miRNA expression in normal, benign, treatment naïve, and castration-resistant prostate samples. The newer version of arrays used in this analysis (v3) contains about 150 more miRNA probes than the previous version of Agilent arrays (v2). Visualisation of all the miRNA raw data generated from these arrays in the form of heatmap revealed that miRNA expression decreases with differentiation in the prostate epithelium. The stem cell population, irrespective of their pathological status, had a higher total miRNA expression than differentiated cells. As miRNAs repress mRNA levels, relatively higher miRNA expression in stem cells should result into relatively lower mRNA levels in stem cells compared to differentiated committed basal cells. This was indeed observed in mRNA microarray analysis performed in our lab in 2008. These differences in total miRNA expression were lost during normalisation, as robust multichip average (RMA) analysis assumes that all the samples have an equal RNA expression and therefore, averages the expression in all the analysed array chips for normalisation (Bolstad et al., 2003). However, even after normalisation, unsupervised heatmap clustered samples clearly on the basis of their differentiation status and not on their pathological status. The differences in expression levels during differentiation were masked in epithelial sub-populations derived from CRPC cultures, suggesting that differentiation is grossly aberrant in CRPCs.

III4.2 Prostate stem cell miRNA signature

We found that 135 miRNAs were significantly differentially expressed in stem and differentiated committed basal cells. This differentiation-influenced cell type-specific miRNA expression pattern has also been noted in a very limited number of studies performed on human tissue-specific stem cells and their differentiated progeny, although, none was as discrete as we have derived (Lee et al., 2011). The prostate

epithelial stem cell miRNA profile mirrored the profile of human embryonic stem cells and other epithelial adult stem cells to some extent (**Table III4.1).** In agreement with previous investigations, miR-143/145, let-7, miR-203, miR-200 family, miR-101, and miR-181a/b were also downregulated in prostate epithelial stem cells in our analysis (Zhang et al., 2010, Cao et al., 2011, Huang et al., 2012, Liu et al., 2012). We have also identified additional miRNAs such as miR-125b (Shi et al., 2010, Zhang et al., 2011) and miR-494 that could potentially regulate epithelial stem cell differentiation (Yang et al., 2008, Dubrovska et al., 2009). Some of the other miRNAs that we have identified have no clear functions ascribed so far, for example miR-10a and miR-766. Exploration of their functions could lead to identification of completely novel regulatory mechanisms active in prostate epithelial differentiation.

MiRNA	Expression	Known function
miR-302 (family)	Upregulated in SC by 2.5-15 fold	Associated with the induction of pluripotency in fibroblasts, to the extent that miR-302 family on its own can transform cancer fibroblasts to induced pluripotent stem cells state (Lin et al., 2008)
miR-145	Downregulated in SC 26.5 fold	Maintenance of prostate, embryonic, corneal and smooth muscle stem cell self-renewal and pluripotency prostate cancer bone metastases and EMT (Cordes et al., 2009, Xu et al., 2009, Lee et al., 2011, Peng et al., 2011, Huang et al., 2012)
Let-7 (Family)	Downregulated in SC by 3-9 fold	Repress 'stemness' by inhibiting self-renewal and promotion of differentiation (Bussing et al., 2008)
miR-8 (family)	Downregulated in SC by 1.5-5 fold	Repress 'stemness' by inhibiting self-renewal and promotion of differentiation (Lin et al., 2009)
miR-17 (family)	Downregulated in SC by 2-12 fold	Repress 'stemness' by inhibiting self-renewal and promotion of differentiation (Foshay and Gallicano, 2009).

Table III4.1: Classical miRNAs that are differentially expressed in stem vs. differentiated CB cells. Multiple investigations show that these miRNA/miRNA families are crucial for human embryonic stem cell maintenance.

III4.3 miRNA characterising prostate cancer

The comparisons between disease association and miRNA expression suggested that changes in miRNA expression during prostate carcinogenesis are subtle, unlike obviously overt changes during differentiation. Even when changes were subtle in some of our comparisons, we obtained consistent and statistically significant results. Most of the miRNAs that were over-expressed in PCa cultures are known to promote tumour growth. For example, miR-886-3p was found to promote cell proliferation and migration in thyroid cancer (Xiong et al., 2011). Investigations in head and neck cancers (Nurul-Syakima et al., 2011) and hepatocellular carcinoma (Lin et al., 2011) showed that miR-1271 and miR-423-3p respectively were upregulated in those cancers compared to normal tissue. Unlike the above mentioned miRNAs, miR-1208 and miR-629 may have a closer and direct relationship with prostate cancer. miR-1208 is located at 8q24, a locus that is frequently mutated in prostate cancer (Witte, 2007). Direct up-regulation of miR-629 by the IL6-STAT3 pathway has been shown to contribute to liver carcinogenesis (Hatziapostolou et al., 2011). This pathway is also frequently active in prostate cancer (Lou et al., 2000) and was shown to be significantly activated in prostate cancer stem cells (Paula Kroon's PhD thesis, 2012) and (Birnie et al., 2008), suggesting a possible role of miR-629 in prostate carcinogenesis. The only significantly downregulated miRNA in PCa was miR-299-5p. This miRNA has been investigated in detail in breast cancer, where it is also under-expressed. The lower expression of miR-299-5p was shown to be necessary for maintaining cell proliferation and metastasis in breast cancer (Shevde et al., 2010, van Schooneveld et al., 2012, Yan et al., 2012). Overall, our analysis has identified several interesting miRNAs that can be instrumental in carcinogenesis. These miRNAs may be performing similar functions to maintain prostate cancer.

III4.4 Prostate cancer stem cell miRNA signature

On further comparison of cancer stem cells enriched from treatment naïve cancers with normal or benign stem cells; a clear prostate cancer stem cell signature was identified. Functional effects of none of these miRNAs have been investigated in detail in relation to cancer stem cell fate to date; but they are predicted to regulate key processes such as (i) chromatin modification (miR-33a*, miR-181a-2*, and miR-532-3p: through HDACs and SMARCs), (ii) cell cycle and proliferation (miR-487b, miR-323-3p and miR-1181: through MAPK and KRAS pathways), (iii) cell adhesion (miR-33a* and miR-181a-2* through cadherins), and (iv) ion transport (miR-181a-2*, miR-411*, and miR-33a* through ABC and Na^{+}-K^{+} transporters) (by miRNA target prediction algorithms). Imbalance in any of these processes is known to cause carcinogenic transformations (Umbas et al., 1992, Johnson et al., 2001, Gottesman et al., 2002, Johnstone, 2002, Wagner and Nebreda, 2009). In a recent 2011 publication, Liu et al identified under-expression of miR-34a in prostate cancer stem cells (Liu et al., 2011). Using cell line models, they showed that overexpression of miR-34a can inhibit CD44 and hence, prostate cancer stem cells. This miRNA is also under-expressed in our analysis in cancer stem cells, but the down-regulation was not statistically significant. Another study by the same group also attempted to identify a prostate cancer stem cell miRNA signature using PPC-1, PC3, LNCaP, and Du145 prostate cell line in addition to LAPC9 xenograft model (Liu et al., 2012). The miRNA signature of cancer stem cells obtained in each of their models was very heterogeneous and the fold changes in differentially expressed miRNAs were minimal. They could identify only 2 differentially expressed miRNAs (overexpression of miR-301 and miR-452 in cancer stem cell population vs. non-cancer stem cell population) common for all the 5 cell types analysed. The observed differences in the cancer stem cell signature in all these models and the subsequent identification of only 2 differentially expressed miRNAs could be predominantly due to analysis of

a limited number of miRNAs (only 310) and inherently very distinct miRNA expression profile cell-types used for analysis. The authors also did not compare their cancer stem cell miRNA signature with a normal or benign stem cell signature. Thus, the cancer stem cell signature in Liu et al study could be a common stem cell miRNA signature and not a specific prostate cancer stem cell miRNA signature.

III4.5 miRNA characterising CRPCs

We have also identified a specific castration-resistant prostate cancer (CRPC) miRNA signature. One of the potential oncogenic candidate miRNA in CRPCs is miR-146a. Investigations in anaplastic thyroid cancer and cervical cancers also found miR-146a to be oncogenic (Wang et al., 2008b, Pacifico et al., 2010). However, in gastric and pancreatic cancers miR-146a is considered as a tumour suppressor (Kogo et al., 2011, Labbaye and Testa, 2012). Xu et al investigated the expression of miR-146a in human CRPCs and noted that miR-146a is down-regulated in a subset of CRPCs (Xu et al., 2012). The authors showed that miR-146a could inhibit the expression of ROCK1 in prostate cell lines, which in turn inhibits cell proliferation and metastasis. These contrasting findings suggest that miR-146a may have tissue specific functions and can influence several signalling pathways. Recent investigations in glioblastoma added extra complexity to the role of miR-146a. Mei and colleagues showed that miR-146a forms a part of an internal feedback regulatory loop to control tumour growth. Activated EGFR signalling and mutated PTEN, which drive glioblastoma growth, can also stimulate miR-146a expression (Mei et al., 2011). Increased miR-146a then inhibited NOTCH1 expression to curtail further growth of the glioblastoma. So even if miR-146a is overexpressed in glioblastoma, it is actually a tumour suppressor miRNA. At least a subset of CRPCs also exhibit high NF-kB activity (Sweeney et al., 2004, Domingo-Domenech et al., 2006), mutated PTEN (Mulholland et al., 2006) and activated

EGFR signalling (Di Lorenzo et al., 2002). Thus, miR-146a sits at the junction of several key pathways that are important in CRPC progression. Its overexpression in the CRPC cultures from our study can signify alterations in any of these or all of these pathways, suggesting that rather than focussing on a one specific target, assessing the genome-wide effects of miRNA expression alteration in a tissue specific manner is vital to obtain the complete spectrum of miRNA functionality.

Another potentially oncogenic miRNA, miR-221, provides more compelling evidence for its oncogenic functions in multiple cancers. Although two investigations indicated the association of miR-221 down-regulation with PCa and CRPC (Ambs et al., 2008, Jalava et al., 2012), other microarray studies and functional investigations showed that miR-221 is necessary for androgen independent growth in vitro and in vivo (Mercatelli et al., 2008, Siva et al., 2009, Sun et al., 2009, Zheng et al., 2012). miR-221 can inhibit $p27^{kip1}$ and can also alter androgen signalling to promote androgen independent growth in prostate cancer (Galardi et al., 2007, Sun et al., 2012). Data from other epithelial cancers, especially breast cancer, indicated that over-expression of miR-221 and down-regulation of the miR-200 family imparts an epithelial to mesenchymal transition (EMT) phenotype (Howe et al., 2012). The over-expression of miR-221 and down-regulation of two miR-200 family members (miR-220b* and miR-200c*) in CRPCs in our analysis imply the occurrence of an EMT in CRPCs. The presence of EMT phenotype is indeed linked to the aggressiveness of multiple cancers, including prostate cancer (Nauseef and Henry, 2011).

miR-1247 has emerged as a prime potential tumour suppressor miRNA from CRPCs in our investigation. This miRNA is usually silenced by DNA methylation in colon cancer (Yan et al., 2011). Ectopic overexpression of miR-1247 in colon cancer cell lines resulted in inhibition of growth and migration (Yan et al., 2011). ADAM15 is one of the important predicted targets of miR-1247, which is considered important

for prostate cancer metastasis (Najy et al., 2008). Identifying the functions of miR-1247 and its relationship with ADAM15 can potentially elucidate miRNA-mediated prostate cancer metastasis pathways. Another miRNA down-regulated in CRPCs is miR-375. It is also consistently down-regulated in lung (Nishikawa et al., 2011), liver (Liu et al., 2010a), cervical (Wang et al., 2011a), head and neck (Avissar et al., 2009), and gastric (Tsukamoto et al., 2010) cancers. In these studies, miR-375 inhibited tumour progression by targeting diverse transcription factors such as, YAP1, SP1, 14-3-3ζ, and PDK1. These findings again illustrates that miRNA can alter multiple pathways and genome-wide correlations should be made to gain complete insight.

III4.6 miRNA-mRNA integrative dataset

The change in miRNA expression, especially during differentiation, was large and involved hundreds of miRNAs. Choosing just a few candidate miRNAs for further evaluation may not elucidate the entire pattern of gene regulation. From the prostate cancer and CRPC signatures, it is clear that multiple miRNAs affect select pathways and processes, e.g. cell fate, chromatin modification, cell adhesion, and ion transport. We reasoned that direct genome-wide correlation between miRNA and mRNA levels would identify signalling pathways principally regulated by miRNAs. Similar integration analyses were performed in prostate and other cancers previously (Enerly et al., 2011). But these studies compared expression of selected miRNAs (usually less than 20) and their mRNA targets in any given tissue.

Our integration analysis revealed that several pathways regulating cell cycle, prostate epithelial-niche interaction, and cell survival could be principally controlled by miRNAs during primary prostate epithelial differentiation. Cell cycle regulation was by far the most common process regulated by miRNAs, suggesting that stem and differentiated committed basal cells have very different cell cycle profiles. It is

indeed noted that adult stem cells in general are relatively quiescent, including prostate epithelial stem cells (Richardson et al., 2004, Collins et al., 2005, Li and Bhatia, 2011). Our integration analysis implies that this quiescence may be mainly regulated by miRNAs in the prostate. This analysis has also identified miRNA-regulated pathways that are instrumental in the regulation of stem cell differentiation, such as estrogen signalling (Hussain et al., 2012) and P38 MAP kinase signalling (Oeztuerk-Winder and Ventura, 2012). Moreover, miRNAs can influence differentiation through regulation of paracrine signalling pathways (IL-1) and niche associated signalling (integrin, BMP, Smad4 and TGF-β pathways). The identification of PI3 kinase, NF-κB, and TRAIL-TNF-α signaling pathways as principal targets of miRNAs in this differentiation associated analysis, might link prostate carcinogenesis with differentiation (An et al., 2003, Paule et al., 2007, Dubrovska et al., 2010). This knowledge can be used to design more specific therapeutic interventions. A more focused approach that can identify key miRNAs affecting several important signaling networks can help to design more specific therapeutic strategies. Several algorithms such as Sigterms, CORNA, and MMIA can help in identification of such key targets (Gunaratne et al., 2010). Thus, our miRNA-mRNA genome wide integration can provide a multi-dimensional view of the regulation of prostate stem cell differentiation.

III4.7 miR-720 as an internal control for qRT-PCR miRNA analysis

Detection of miRNA expression by qRT-PCR is the commonest technique used for the analysis of miRNA expression. In order to normalise expression of miRNAs in multiple samples, a good internal control (or reference gene) is essential. Recent reports suggest that RNU6b may not be an ideal internal control as it has some functional roles, e.g. regulation of cell proliferation, and can endure a significant

change in expression in cancer, more notably in urological cancers (Wotschofsky et al., 2011, Ratert et al., 2012). It would be ideal to identify a miRNA with high expression and low variance between samples to be analysed by qRT-PCR to use as an internal control. Therefore, we looked into miRNAs that had high expression and low variance among all the miRNA samples. miR-720 had the highest expression and least variance. After ruling out the prostate specificity of miR-720, we confirmed that miR-720 could be a better internal control for miRNA qRT-PCR analysis on primary prostate epithelial cultures. This is the first study that identifies a particular miRNA as a suitable internal control for miRNA-related primary prostate epithelial investigations.

In summary, this miRNA analysis has created a statistically significant and consistent miRNA microarray database. This database could be enhanced by adding the normal and cancerous luminal cell miRNA profile. Together with the integrated mRNA database, novel and specific targets for diagnosis/prognosis and management of prostate cancer could now be identified.

4. CONCLUSIONS

The main purpose of this investigation was to delineate critical pathways, which are responsible for the maintenance of prostate epithelial hierarchy in patient-derived prostate tissue. Three distinct but converging approaches were used: a transcriptional regulatory approach, telomerase-mediated regulation, and miRNA-mediated microarray analyses have identified novel regulatory mechanisms. The output of these investigations not only provided direct information about basic pathophysiology of human adult prostate epithelial stem cell maintenance but can also potentially be used for diagnostic, prognostic, and therapeutic purposes.

Multiple lines of evidence in our analyses suggested that prostate cancer stem cells (CSCs) are quiescent. The foremost evidence came from the observation of undetectable telomerase expression and activity in prostate CSCs. This observation is supported by independent finding of significantly lower Ki-67 immunostaining in prostate CSCs compared to their differentiated progeny (Frame et al., in preparation). Investigations from other labs also suggested that prostate CSCs or cells similar to prostate CSCs in their models could be quiescent (Palapattu et al., 2009, Qin et al., 2012). The interesting fact is that the differentiated progeny of prostate cancer stem cells (transit amplifying cells, committed basal cells, and luminal cells) express relatively high levels of telomerase, indicating a proliferative nature. There could be three possible explanations for this differential telomerase expression. First, the differentiated prostate cancer cells lose contact with stromal niche. TGF-β signalling, one of the pathways in this niche, is known to directly regulate hTERT expression (Tsujimura et al., 2002, Geserick et al., 2006, Cassar et al., 2010). Changes in similar stromal niche influences can therefore dictate differential telomerase

expression. Second, there could be differential post-transcriptional or epigenetic regulation of hTERT or one of its critical interaction partners. Our miRNA-mRNA analysis has indeed showed that pathways regulating cell quiescence and proliferation (including TGF-β signalling) could be under strict miRNA control (Table III3.5). And third, there could be a change in expression of one of the critical hTERT interaction partners or transcriptional factors regulating hTERT expression during differentiation. For example, WNT and hTERT are likely to be involved in a negative feedback loop (Park et al., 2009, Hoffmeyer et al., 2012) and one of the WNT ligand (WNT5a) is differentially expressed between prostate cancer stem cells and their differentiated progeny (Birnie et al., 2008), which may influence hTERT functionality. Similar mechanisms may also be responsible for differential telomerase expression in sub-populations enriched from benign prostatic hyperplasia. Further exploration of these possibilities is essential to determine more precise regulation of prostate cancer stem cell quiescence and proliferative potential of other hierarchical sub-populations.

The majority of the remaining investigations were focused on prostate stem cell differentiation. These investigations indicated that prostate stem cell differentiation could be a process delicately controlled by a mRNA-miRNA network. The data based on mRNA microarrays provided a compelling initial evidence for a retinoic acid regulated network of LCN2, CEACAM6, and S100p in the regulation of prostate epithelial stem cells enriched from BPH and treatment naïve prostate cancer-derived cultures. One additional feature of the LCN2, CEACAM6, and S100p gene group is that they all can be regulated by a common group of 24 transcription factors, most of which can in turn be regulated by only three miRNAs (miR128, miR-188, and miR-548c). This observation suggested that these 3 miRNAs along with LCN2, CEACAM6, and S100p could be important for the regulation of prostate epithelial stem cell differentiation.

Apart from this, prostate epithelial stem cells also exhibit a classical human embryonic stem cell-like miRNA signature in addition to a prostate stem cell-specific miRNA signature. All this information suggested that several genes and miRNAs participate in the regulation of prostate epithelial stem cell differentiation. Thus, choosing just one or two 'promising' target genes/miRNAs may not capture the central regulatory circuitry in its entirety. We have created a genome-wide integrative dataset composed of miRNAs over- or under-expressed in stem cells, compared to their differentiated progeny, and related this to the correspondingly inversely expressed mRNA targets of these miRNAs to provide a powerful and unbiased approach for the identification of novel pathways responsible for prostate epithelial stem cell maintenance. This integrative dataset has the ability to identify key nodes (miRNAs and mRNAs) that can be targeted to perturb prostate epithelial stem cell regulation, which can potentially be exploited to develop specific and efficient prostate stem cell targeting therapies.

Finally, a note on the prostate epithelial hierarchy in castration-resistant prostate cancer (CRPC) and patient-derived prostate xenograft in immunocompromised mice. Our investigations of the expression of the candidate genes (LCN2, CEACAM6, and S100p) and telomerase consistently showed that their expression magnitude and pattern was noticeably different in the prostate epithelial sub-populations enriched from CRPCs and patient-derived prostate xenografts, compared to BPH or treatment naïve prostate cancer-derived sub-populations. Even the miRNA expression pattern in sub-populations enriched from CRPCs was remarkably different. These findings suggest that the prostate epithelial hierarchy is significantly disorganised in CRPCs and xenografts and/or a modified set of surface-markers is necessary to enrich pure and homogeneous hierarchical sub-populations from these two systems. In any case, our findings

suggest that the CRPCs and patient derived xenografts are more similar to each other, but differ remarkably from treatment naïve prostate cancers.

In summary, we have identified potentially novel miRNA and mRNA-mediated prostate stem cell regulatory pathways, in addition to the identification of cell and microenvironment specific telomerase expression pattern in the prostate epithelial sub-populations.

5. APPENDICES

Appendix 1:

Top 50 genes, which are significantly overexpressed in committed basal cells (CB) compared to stem cells (SC). Expression mean is mentioned as log2.

	Symbol	Description	mean (SC)	mean (CB)	difference in means
1	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	6.99151	10.0672	3.07568
2	SPRR1A	small proline-rich protein 1A	8.10551	11.1592	3.05369
3	SPRR3	small proline-rich protein 3	6.91313	9.891	2.97787
4	CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	6.05929	8.94361	2.88433
5	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	7.43453	10.2339	2.79939
6	RARRES1	retinoic acid receptor responder (tazarotene induced) 1	4.16271	6.87451	2.7118
7	KRT13	keratin 13	7.11128	9.76094	2.64965
8	S100P	S100 calcium binding protein P	8.62272	11.177	2.55424
9	CALB1	calbindin 1, 28kDa	5.46743	7.96813	2.50069
10	SPINK7	serine peptidase inhibitor, Kazal type 7 (putative)	5.95419	8.32059	2.3664
11	CALB1	calbindin 1, 28kDa	6.24066	8.56515	2.32449
12	SLC6A14	solute carrier family 6 (amino acid transporter), member 14	8.57649	10.8768	2.30031
13	S100A7	S100 calcium binding protein A7	5.20657	7.47894	2.27237
14	CEACAM7	carcinoembryonic antigen-related cell adhesion molecule 7	4.95722	7.22583	2.2686
15	PIP	prolactin-induced protein	6.39722	8.66163	2.2644
16	GCNT3	glucosaminyl (N-acetyl) transferase 3, mucin type	6.72717	8.98091	2.25374
17	SERPINB3	serpin peptidase inhibitor,	8.01811	10.2594	2.24127

		clade B (ovalbumin), member 3			
18	SERPINB3	serpin peptidase inhibitor, clade B (ovalbumin), member 3	8.78593	11.0193	2.23342
19	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	5.80864	8.03769	2.22904
20	SERPINB4	serpin peptidase inhibitor, clade B (ovalbumin), member 4	4.35602	6.5467	2.19068
21	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	4.9533	7.02639	2.07309
22	HOPX	HOP homeobox	7.0089	9.04912	2.04022
23	RARRES1	retinoic acid receptor responder (tazarotene induced) 1	3.91068	5.93452	2.02384
24	DHRS9	dehydrogenase/reductase (SDR family) member 9	7.8965	9.88951	1.99302
25	DHRS9	dehydrogenase/reductase (SDR family) member 9	7.84488	9.83075	1.98587
26			7.34314	9.25366	1.91052
27	CRCT1	cysteine-rich C-terminal 1	6.86493	8.74567	1.88074
28	SERPINB13	serpin peptidase inhibitor, clade B (ovalbumin), member 13	5.7174	7.59684	1.87944
29	PI3	peptidase inhibitor 3, skin-derived	8.72965	10.5821	1.85249
30	SPRR3	small proline-rich protein 3	6.27342	8.11858	1.84515
31	SPRR1A	small proline-rich protein 1A	8.49782	10.3273	1.8295
32	LCN2	lipocalin 2	9.38905	11.1813	1.79226
33	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	7.48053	9.24031	1.75978
34	PI3	peptidase inhibitor 3, skin-derived	8.24627	10.0002	1.75397
35	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	4.06631	5.8156	1.74928
36	SPRR1B	small proline-rich protein 1B (cornifin)	9.74324	11.4762	1.73293
37	TMEM45B	transmembrane protein 45B	6.97697	8.68186	1.70489
38	SERPINB13	serpin peptidase inhibitor, clade B (ovalbumin), member 13	5.21592	6.89262	1.6767
39	NEBL	nebulette	5.07518	6.6585	1.58332
40	DHRS9	dehydrogenase/reductase	9.95676	11.5048	1.54799

		(SDR family) member 9			
41	CXCL17	chemokine (C-X-C motif) ligand 17	6.25709	7.79884	1.54175
42	AGR2	anterior gradient homolog 2 (Xenopus laevis)	6.54235	8.04734	1.50499
43	SCEL	sciellin	7.30742	8.81141	1.50399
44	GDF15	growth differentiation factor 15	7.82598	9.31014	1.48416
45	KLK7	kallikrein-related peptidase 7	7.24742	8.72544	1.47803
46	CAPN14	calpain 14	4.00898	5.48083	1.47185
47	MUC20	mucin 20, cell surface associated	5.4347	6.8867	1.452
48			5.67062	7.12034	1.44972
49	MAL	mal, T-cell differentiation protein	5.13578	6.5784	1.44263
50	KLK7	kallikrein-related peptidase 7	7.55934	8.98312	1.42379

Appendix 2

miRNA significantly upregulated in stem cells (SC) compared to differentiated committed basal cells (CB): Fold difference >3, mean expression > 20 arbitrary units in microarrays, and p > 0.01, t-test: paired two-tailed t-test and u-test: Wilcoxon rank-sum test.

	miRNA	p (t-test)	p (u-test)	SC/CB
1	hsa-miR-513a-5p	0.003627	0.000125	207.6097
2	hsa-miR-572	0.00011	7.47E-06	98.84424
3	hsa-miR-638	2.48E-05	7.47E-06	95.58457
4	hsa-miR-765	0.005706	2.14E-05	85.85846
5	hsa-miR-663	0.001224	0.000125	76.67911
6	hsa-miR-1321	0.001839	7.02E-05	72.03593
7	hsa-miR-345	0.003128	0.00018	55.84791
8	hsa-miR-630	4.92E-05	9.25E-06	47.3539
9	hsa-miR-483-5p	0.006555	0.004717	42.91736
10	hsa-miR-648	0.0044	7.04E-05	38.39434
11	hsa-miR-1915	0.000224	7.47E-06	33.91599
12	hsa-miR-494	9.51E-05	0.000125	33.67538
13	hsa-miR-940	0.001041	7.47E-06	29.71811
14	hsa-miR-1826	0.000409	0.000125	26.76884
15	hsa-miR-1246	0.000353	9.25E-06	26.02523
16	hsa-miR-566	0.008334	0.001926	23.26418
17	hsa-miR-874	0.001973	0.000125	21.43633
18	hsa-miR-1207-5p	0.000124	0.000125	18.54786
19	hsa-miR-1228	0.00789	0.000103	16.63887
20	hsa-miR-631	0.006428	0.001198	13.79996
21	hsa-miR-766	0.000975	7.47E-06	13.28816
22	hsa-miR-1275	0.000716	0.000181	13.07876
23	hsa-miR-617	4.72E-06	7.47E-06	12.64253
24	hsa-miR-92b	0.007817	1.14E-05	12.62047
25	hsa-miR-636	0.00394	9.25E-06	12.30161
26	hsa-miR-574-5p	0.000295	7.47E-06	12.12288
27	hsa-miR-220c	0.003996	2.35E-05	11.71661
28	hsa-miR-653	0.004335	0.00091	10.67538
29	hsa-miR-1825	0.004024	7.47E-06	10.62787
30	hsa-miR-634	0.00836	2.62E-05	9.161396
31	hsa-miR-623	0.001985	3.92E-05	9.006261
32	hsa-miR-662	0.001176	0.004082	8.602362
33	hsa-miR-1290	0.006363	0.007189	8.346788
34	hsa-miR-939	0.000445	0.000125	7.664512
35	hsa-miR-1272	0.000864	0.002618	7.618045
36	hsa-miR-659	0.001258	0.000522	7.318321
37	hsa-miR-1202	0.000908	0.000259	5.476692

38	hsa-miR-935	0.000895	0.001235	4.892814
39	hsa-miR-665	0.001007	1.14E-05	4.315723

Appendix 3:

miRNA significantly upregulated in differentiated committed basal cells (CB) compared to stem cells (SC): Fold difference >3, mean expression > 20 arbitrary units in microarrays, and p > 0.01, t-test: paired two-tailed t-test and u-test: Wilcoxon rank-sum test

	Mir name	p (t-test)	p (u-test)	CB/SC
1	hsa-miR-153	3.71E-08	3.82E-06	169676.6
2	hsa-miR-143	2.80E-08	7.34E-06	137.9731
3	hsa-miR-340	4.22E-09	7.32E-06	124.7923
4	hsa-miR-335	1.79E-07	7.26E-06	118.2251
5	hsa-miR-532-3p	1.13E-09	7.38E-06	91.92148
6	hsa-miR-542-3p	1.91E-08	7.47E-06	54.82151
7	hsa-miR-652	2.25E-11	7.30E-06	40.71522
8	hsa-miR-218	1.68E-10	7.47E-06	40.70796
9	hsa-miR-152	5.36E-09	7.45E-06	39.07096
10	hsa-miR-362-3p	3.28E-09	7.47E-06	36.42715
11	hsa-miR-181c	2.41E-09	7.40E-06	36.41657
12	hsa-miR-194	3.92E-10	7.47E-06	34.42391
13	hsa-miR-195	3.53E-11	7.47E-06	32.55667
14	hsa-miR-199b-5p	1.45E-09	7.47E-06	29.75735
15	hsa-miR-532-5p	1.59E-10	7.47E-06	29.58983
16	hsa-miR-145	1.19E-08	7.47E-06	24.66274
17	hsa-miR-132	2.67E-09	7.38E-06	23.77544
18	hsa-miR-450a	4.92E-09	7.47E-06	23.63056
19	hsa-miR-214	4.33E-10	7.38E-06	23.09888
20	hsa-miR-219-5p	7.72E-09	7.47E-06	22.89941
21	hsa-miR-744	1.15E-09	7.47E-06	22.68216
22	hsa-miR-193a-3p	1.09E-08	7.47E-06	22.46801
23	hsa-miR-10a	1.17E-10	7.47E-06	22.119
24	hsa-miR-199a-5p	2.31E-12	7.47E-06	21.73395
25	hsa-miR-301a	8.60E-14	7.47E-06	21.38827
26	hsa-miR-30e	1.80E-12	7.47E-06	20.80675
27	hsa-miR-25	6.22E-10	7.47E-06	19.9304
28	hsa-miR-34c-5p	5.60E-11	7.47E-06	19.91615
29	hsa-miR-497	1.16E-10	7.45E-06	19.55311
30	hsa-miR-582-5p	7.56E-10	7.47E-06	19.53977
31	hsa-miR-106b	1.69E-10	7.47E-06	19.0844
32	hsa-miR-101	7.20E-18	7.47E-06	19.07501
33	hsa-miR-10b	1.81E-09	7.47E-06	17.49733
34	hsa-miR-185	5.85E-16	7.47E-06	17.49473
35	hsa-miR-93	1.18E-10	7.47E-06	17.23883

36	hsa-miR-128	2.29E-11	7.45E-06	16.58712
37	hsa-miR-30a*	9.87E-13	7.45E-06	16.57182
38	hsa-miR-33a	3.95E-10	7.47E-06	15.98261
39	hsa-miR-99b	5.18E-10	7.47E-06	15.56193
40	hsa-miR-99a	4.06E-13	7.47E-06	15.24409
41	hsa-miR-28-5p	4.61E-15	7.47E-06	14.66839
42	hsa-miR-32	1.78E-09	7.47E-06	14.09838
43	hsa-miR-374b	8.33E-15	7.47E-06	13.77631
44	hsa-miR-30a	8.37E-16	7.47E-06	13.02261
45	hsa-miR-30c	1.47E-07	7.47E-06	12.42804
46	hsa-miR-148b	7.16E-16	7.47E-06	12.06068
47	hsa-miR-18a	1.64E-12	9.25E-06	11.95384
48	hsa-miR-542-5p	7.07E-09	7.43E-06	11.43153
49	hsa-miR-324-5p	3.67E-12	7.47E-06	11.35735
50	hsa-miR-30b	6.52E-17	7.47E-06	11.23044
51	hsa-miR-342-3p	3.65E-13	9.25E-06	10.87114
52	hsa-miR-96	2.04E-11	7.47E-06	10.86863
53	hsa-miR-151-5p	6.23E-16	7.47E-06	10.84536
54	hsa-miR-126	8.20E-13	7.47E-06	10.55507
55	hsa-miR-502-3p	1.62E-10	7.47E-06	10.45076
56	hsa-miR-98	8.34E-13	7.47E-06	10.42639
57	hsa-miR-17*	1.01E-10	1.14E-05	10.3463
58	hsa-let-7i	7.33E-11	7.47E-06	9.234769
59	hsa-miR-183	3.08E-11	9.25E-06	8.676576
60	hsa-miR-130a	9.11E-15	7.47E-06	8.389286
61	hsa-miR-378	1.15E-12	7.47E-06	8.155129
62	hsa-miR-130b	2.41E-10	9.25E-06	7.929966
63	hsa-miR-15b	2.04E-12	7.47E-06	7.504631
64	hsa-miR-425	4.90E-10	9.23E-06	7.02421
65	hsa-miR-186	1.34E-11	7.47E-06	6.991253
66	hsa-miR-16	4.78E-12	7.47E-06	6.707356
67	hsa-let-7e	1.85E-11	7.47E-06	6.6934
68	hsa-miR-18b	1.39E-10	7.47E-06	6.400111
69	hsa-miR-125b	3.91E-15	7.47E-06	6.118902
70	hsa-let-7d	4.89E-14	7.47E-06	6.102874
71	hsa-miR-26a	3.78E-14	7.47E-06	5.675824
72	hsa-miR-224	3.28E-11	7.47E-06	5.613235
73	hsa-miR-26b	3.09E-12	7.47E-06	5.55756
74	hsa-miR-361-5p	1.37E-09	2.14E-05	5.479726
75	hsa-miR-487b	1.60E-07	2.62E-05	5.35076
76	hsa-miR-500*	1.57E-10	7.47E-06	5.278184
77	hsa-miR-19a	1.32E-10	7.47E-06	5.197589
78	hsa-miR-29a	1.24E-15	7.47E-06	5.041888
79	hsa-miR-20b	1.00E-11	7.47E-06	4.930728

80	hsa-miR-21	1.09E-11	9.25E-06	4.810083
81	hsa-miR-423-5p	3.24E-10	7.47E-06	4.62981
82	hsa-miR-182	1.20E-08	3.92E-05	4.611759
83	hsa-miR-29c	7.33E-11	7.47E-06	4.393718
84	hsa-let-7g	1.08E-10	7.47E-06	4.30273
85	hsa-miR-221*	3.60E-07	7.05E-05	4.19019
86	hsa-miR-29b	1.23E-11	7.47E-06	4.176767
87	hsa-miR-21*	1.88E-09	9.25E-06	4.003028
88	hsa-miR-107	1.08E-08	9.25E-06	3.936753
89	hsa-miR-15a	6.28E-09	2.62E-05	3.814922
90	hsa-miR-22	2.27E-09	1.14E-05	3.762419
91	hsa-miR-34b*	1.53E-08	2.62E-05	3.718489
92	hsa-miR-210	7.45E-09	1.74E-05	3.649707
93	hsa-miR-31	4.53E-11	7.47E-06	3.485603
94	hsa-miR-181b	1.14E-09	1.41E-05	3.268455
95	hsa-miR-23b	7.74E-08	1.41E-05	3.254016
96	hsa-miR-27b	1.62E-08	1.74E-05	3.23319
97	hsa-miR-212	5.71E-09	1.41E-05	3.178387
98	hsa-miR-222	2.74E-07	4.78E-05	3.097119
99	hsa-miR-19b	1.06E-07	1.14E-05	3.041002

Appendix 4:

miRNAs with high expression and low variations

miRNA	Mean expression (log 10)	Variance
hsa-let-7a	3.320	0.116
hsa-let-7b	3.176	0.042
hsa-let-7c	2.919	0.098
hsa-miR-15a	2.276	0.142
hsa-miR-34a	2.407	0.101
hsa-miR-221	2.443	0.125
hsa-miR-125a-5p	2.047	0.194
hsa-miR-365	2.403	0.083
hsa-miR-324-3p	2.199	0.049
hsa-miR-320b	2.072	0.115
hsa-miR-1202	2.321	0.170
hsa-miR-1260	3.407	0.099
hsa-miR-1274b	3.856	0.136
hsa-miR-1280	2.299	0.089
hsa-miR-1308	2.320	0.107
hsa-miR-720	4.239	0.040
hsa-miR-320d	2.039	0.073

6. ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometre
5-Azt	5-Aza-2'-deoxycytidine
ADT	Androgen deprivation therapy
ALT	Alternative lengthening of telomeres
AR	Androgen receptor
ARHI	Aplasia Ras homolog member I
ATCC	American type culture collection
at-RA	All-*trans* retinoic acid
AZ	Anterior Zone
BCA	Bicinchoninic acid
bp	Base pairs
BPH	Benign Prostatic Hyperplasia
BSA	Bovine serum albumin
CB	Committed basal
CD	Cluster of differentiation
cDNA	Complimentary DNA
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
ChIP	Chromatin Immunoprecipitation
CK	Cytokeratin
CO_2	Carbon dioxide
CpG	Cytosine-phosphate-guanine
CRPC	Castrate resistant prostate cancer
CR-PCa	Castrate resistant prostate cancer
CRU	Cancer research unit
CSC	Cancer stem cell
C_t	Threshold cycle
CTC	Circulating tumour cell
CZ	Central zone
D10	DMEM + 10% FCS
DAPI	4',6-diamidino-2-phenylindole
ddH_20	Double distilled water
dH_2O	Distilled water
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPS	Deoxyribonucleotide triphosphate
ECACC	European collection of cell cultures
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMBOSS	European molecular biology open software suite
EMT	Epithelial-to-mesenchymal transition
ERG	V-ets erythroblastosis virus E26 oncogene homolog
ES	Embryonic stem
EtOH	Ethanol

FCS	Fetal calf serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FISH	Florescent in situ hybridisation
g	Gram
G0	G zero phase
G1	Gap 1 phase
G2/M	Gap 2 phase/mitosis
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotrophin-releasing hormone
G_Y	Gray
H7	Ham's F-12 medium + 7% FCS
HCl	Hydrogen chloride
HDAC	Histone deacetylase
hES	Human embryonic stem
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA
IgG	Immunoglobulin G
kB	Kilobase
KCl	Potassium chloride
kDa	Kilo Dalton
Ki67 (MKI67)	Antigen identified by monoclonal antibody Ki-67
KSFM	Keratinocyte serum free medium
LA	Left apex
LB	Left base
LCN2	Lipocalin 2
LIF	Leukaemia inhibitory factor
LRP	Laparoscopic radical prostatectomy
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium chloride
Min	Minute
MiRNA	Micro RNA
ml	Millilitre
mM	Millimolar
mm	millimetre
MMP	Matrix metallopeptidase
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ND	Non-detectable
ng	Nanogram
NKX3.1	NK3 transcription factor related, locus 1
nM	Nanomolar
NP-40	Tergitol-type NP-40
P21	Cyclin-dependent kinase inhibitor 1A
P27KIP	Cyclin-dependent kinase inhibitor 1B(CDKN1B)
P53	Tumour protein 53
P63	Tumour protein 63
PAGE	Polyacrylamide gel electrophoresis
PAP	Prostatic acid phosphatase
PBS	Phosphate-buffered saline
PCa	Treatment naïve prostate cancer
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase

PIN	Prostatic intraepithelial neoplasia
PSA	Prostate-specific antigen
PSAPb	PSA-probasin
qPCR	Quantitative PCR
qRT-PCR	Quantitative real-time PCR
R10	RPMI + 10% FCS
R1881	Metribolone
R5	RPMI + 5% FCS
RA	Retinoic acid
Rag2	Recombination activating gene 2
RAR	Retinoic acid receptor
RARα	RAR alpha
RARβ	RAR beta
RARγ	RAR gamma
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPLP0	Ribosomal protein, large, P0
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RXR	Retinoid X receptor
RARα	RXR alpha
RARβ	RXR beta
RARγ	RXR gamma
S100P	S100 calcium binding protein P
SC	Stem cell
SCM	Stem cell medium
Scr	Scrambled siRNA
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SPRR3	Small proline-rich protein 3
SV40	Simian vacuolating virus 40 Tag
TA	Transit-amplifying cell
TAE	Tris base, acetic acid and EDTA buffer
Taq	Thermus aquaticus
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TE	Tris EDTA buffer
TERT	Telomerase reverse transcriptase
TF	Transcription factor
TMPRSS2	Transmembrane protease, serine 2
TR	Telomerase RNA
TRAP	Telomeric repeat amplification protocol
TSA	Trichostatin A
TSS	Transcription start site
TURP	Transurethral resection of the prostate
TX-100	Triton X-100
TZ	Transitional zone
UK	United Kingdom
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WNT	Wingless-type MMTV integration site family
β-actin	Beta actin

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